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01/19/00 09/487023

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	21327-701-01 US	*Total Pages*	36
First Named Inventor or Application Identifier	Parkash S. Gill		
Express Mail Label No.	EL128286694US		

01/19/00 09/487023

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents:

Assistant Commissioner for Patents

ADDRESS TO: **Box Patent Application**
Washington, DC 20231

1. [X] Fee Transmittal Form
(Submit an original, and a duplicate for fee processing)

2. [X] Specification (with cover sheet) [Total Pages [36]]
(preferred arrangement set forth below)
- Descriptive title of the Invention
- Cross References to Related Applications
- Statement Regarding Fed sponsored R & D
- Reference to Microfiche Appendix
- Background of the invention
- Brief Summary of the Invention
- Brief Description of the Drawings (if filed)
- Detailed Description
- Claim(s)
- Abstract of the Disclosure

3. [X] Drawings (35 USC 113) [Total Sheets [19]]

4. Oath or Declaration [Total Pages [3]]

a. [X] Newly executed (original or copy) **(UNSIGNED)**

b. [ ] Copy from a prior application (37 CFR 1.63(d))
(for continuation/divisional with Box 17 completed)
[Note Box 5 below]
1. [ ] DELETION OF INVENTOR(S)
Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b)

5. [ ] Incorporated By Reference (useable if Box 4b is checked) The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

6. [ ] Microfiche Computer Program (Appendix)

7. Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)
a. [ ] Computer Readable Copy
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c. [ ] Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. [ ] Assignment Papers (cover sheet & document(s))

9. [ ] 37 CFR 3.73(b) Statement (when there is an assignee) [ ] Power of Attorney

10. [ ] English Translation Document (if applicable)

11. [ ] Information Disclosure Statement (IDS)/PTO-1449 [ ] Copies of IDS Citations

12. [ ] Preliminary Amendment

13. [X] Return Receipt Postcard (MPEP 503)
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14. [X] Small Entity Statement(s) [ ] Statement filed in prior application, Status still proper and desired

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17. **If a CONTINUING APPLICATION,**
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[ ] Continuation [ ] Divisional [X] Continuation-in-part (CIP)

of prior application No: 09/016,541

18. CORRESPONDENCE ADDRESS

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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Gill, Parkash and Masood, Rizwan
Application No.:	Unknown
Filed or Issued:	Herewith
For:	METHOD AND COMPOSITION FOR TREATMENT OF KAPOSI'S SARCOMA

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 C.F.R. §§ 1.9(f) AND 1.27(b)) - INDEPENDENT INVENTOR

As below named inventors, we hereby declare that we qualify as independent inventors as defined in 37 C.F.R. § 1.9(c) for purposes of paying reduced fees under Section 41 (a) and (b) of Title 35, United States Code, to the United States Patent and Trademark Office with regard to the invention entitled METHOD AND COMPOSITION FOR TREATMENT OF KAPOSI'S SARCOMA.

described in

(X) the specification filed herewith
( ) application Serial No. [ ], filed [Date]
( ) Patent No. [ ], issued [Date].

we have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not likewise be classified as an independent inventor under 37 C.F.R. § 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 C.F.R. § 1.9(d) or a nonprofit organization under 37 C.F.R. § 1.9(e).

Each person, concern or organization to which we have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

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We hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

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Date: _____

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Signature: _____

Date: _____

APPLICATION

FOR

UNITED STATES PATENT

on

METHOD AND COMPOSITION FOR TREATMENT OF KARPOSI'S SARCOMA

by

Parkash S. Gill
Rizwan Masood

Sheets of Drawings: 19

Docket No.: 21327-701-01 US

Michael J. Shuster, Ph.D.
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* * * * * *

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICATION FOR PATENT ON

METHOD AND COMPOSITION FOR TREATMENT OF KAPOSI'S SARCOMA

by

Parkash S. Gill

Rizwan Masood

1. RELATED APPLICATION

This application is a continuation-in-part of U.S. Application No. 09/016,541 filed January 30, 1998, which claims the benefit of U.S. Provisional Application No. 60/037,004, filed January 31, 1997, the disclosures of which are both incorporated by reference in their entirety.

2. FIELD OF INVENTION

The invention relates to the pharmacological use of antisense oligonucleotides directed against vascular endothelial growth factor (VEGF). Antisense VEGF inhibitors can be used in the treatment of Kaposi's sarcoma (KS) in patients by administering to a patient an effective amount of an antisense oligonucleotide which is capable of inhibiting the growth of KS cells in culture. The invention also relates to the use of antisense VEGF inhibitors in the treatment of other diseases in which vascular proliferation plays a role, such as cancers, particularly in the treatment of ovarian cancer, pancreatic carcinoma and melanoma.

3. BACKGROUND OF INVENTION

Kaposi's sarcoma (KS) is the most common tumor seen in patients with HIV-1 infection (Lifson *et al.*, 1990; Reynolds, P. *et al.*, 1993). KS causes significant morbidity and mortality through involvement of the skin and visceral organs. While the etiologic agent, if any, is unknown, substantial knowledge has been gained regarding the factors regulating the growth of tumor cells (Reynolds *et al.*, 1993).

Kaposi's sarcoma most frequently presents as skin lesions (Lifson *et al.*, 1990). Mucosal (oral cavity) involvement is the second most common site of disease, occurring on the palate and gums and can cause tooth loss, pain and ulceration. Lymph node involvement is common with KS. However, the precise frequency is not known due to the lack of routine lymph node biopsies.

Visceral involvement occurs frequently, (in nearly 50% of the cases) especially in patients with advanced disease (Laine, L. *et al.*, 1987). Advanced gastrointestinal (GI) KS can cause enteropathy, diarrhea, bleeding, obstruction and death. Pulmonary involvement is common and significant pulmonary KS occurs in nearly 20% of the cases (Laine *et al.*, 1987; Gill, P.S. *et al.*, 1989). The symptoms vary from no symptoms to dry cough, exertional dyspnea, hemoptysis and chest pain. Pulmonary function studies may show varying degree of hypoxemia. The overall survival of patients with symptomatic pulmonary KS is less than 6 months (Gill *et al.*, 1989).

While the skin, lung, and GI tract are common sites of disease, nearly every organ can be involved with KS, including liver, spleen, pancreas, omentum, heart, pericardium, etc.

Phenotypic studies to define the cell of origin of KS have been performed extensively. KS spindle cells express phenotypic features of mesenchymal cells and share some markers with endothelial cell, vascular smooth muscle cells, and dermal dendrocytes. The markers shared with endothelial cells include lectin binding sites for *Ulex europeaus* Agglutinin-1 (UEA-1), CD34, EN-4, and PAL-E. The expression of several factors markers in human umbilical vein endothelial cells (HUVEC), AIDS-KS cells and trans differentiated HUVEC was confirmed by histochemistry and RT-RCR message analysis for expression of IL-6, IL-8, GM-CSF, TGF-β etc.

AIDS-KS spindle cell isolation has allowed the determination of factors secreted by the tumor cells and their effects on the tumor cell itself. Both IL-1 β and IL-6 are produced by tumor cells. Further, the inhibition of their effects either through blocking their binding to the cognate receptors (IL-1 receptor antagonist, soluble IL-1 receptor) or inhibition of gene expression through antisense oligonucleotides (for IL-6) inhibits the growth of tumor cells. More importantly, both IL-1 and IL-6 induce VEGF expression. Thus endogenous production of these factors may in part be responsible for high levels of VEGF production by KS cells.

VEGF was first discovered as a molecule that is a secreted protein with biological effects which include the following: VEGF *in vitro* induces the growth of endothelial cells and induces migration of endothelial cells; VEGF induces new vessel formation in model systems, such as the chick chorioallantoic membrane and the rat or rabbit cornea avascular zone; VEGF induces permeability of the existing blood vessels, in model systems, such as the mice of guinea pig skin vessels. It was later shown that a number of tumor cells produce VEGF and the secreted protein induces the regional blood vessels to produce more blood vessel network to support the tumor growth and metastasis. In addition, inhibition of VEGF function was shown to reduce the growth potential of tumor explants in immunodeficient mice. VEGF expression is increased by hypoxia as noted in the deepest part of the tumor, and by certain cytokines, such as IL-1 and IL-6. VEGF functions through the cognate tyrokinase receptors, Flt-1 and Flk-1/KDR. Flt-1 is an intermediate affinity receptor and Flk-1/KDR is a low affinity receptor. Expression of both receptors results in high affinity binding of the homodimer of VEGF to the target cells. Signal transduction, however, occurs through Flk-1/KDR only.

VEGF is expressed as four different splice variants. VEGF 165 and VEGF 121 are secreted proteins. Four other members of the VEGF family have been described recently. These include VEGF-B, VEGF-C, VEGF-D, and placental derived growth factor (PIGF). KS cells express all members of the VEGF family, as well as the receptors for VEGF and VEGF-C (Flt-4). PIGF has 47% homology to VEGF and binds to Flt-1 as a homodimer or a heterodimer with VEGF. VEGF-B is a 167 amino acid secreted protein and has 43% and 30% homology with VEGF and PIGF. VEGF-C also called VEGF related protein (VRP) has 32% and 27% homology to VEGF and PIGF. It binds to Flt-4 as a homodimer and to Flk-1/KDR as a VEGF heterodimer.

The hallmark of KS is the aberrant and enhanced proliferation of vascular structures. Various angiogenic factors have been isolated for their ability to enhance endothelial cell proliferation and migration *in vitro*. Analysis of AIDS-KS cells has revealed the expression of basic fibroblast growth factor (bFGF) and vascular endothelial cell growth factor (VEGF). The latter is a secreted molecule with capability to induce capillary permeability, a prominent feature of a subset of AIDS-KS. Inhibition of VEGF expression may have therapeutic efficacy in KS. In addition, the isolation of several members of the VEGF family reveals that there is a redundancy

and modulation of VEGF function. It is thus conceivable that the inhibition of VEGF alone may be active as a therapeutic strategy to inhibit tumor growth, while inhibition of several or all members of this family may be more effective.

The treatment of AIDS-related Kaposi's sarcoma is palliative. Localized KS can be managed with local therapy including radiation therapy. Radiation therapy produces local toxicity and has a cumulative dose limiting toxicity. Other options for the cosmetic treatment of localized disease include cryotherapy, photodynamic therapy, intralesional vinblastine, and intralesional sclerosing agents, all of which result in local toxicity or pigmentation which may at times be worse than the lesions itself.

Progressive KS especially with local complications of pain, edema, and ulceration and symptomatic visceral KS, requires therapy which will result in rapid response. Systemic cytotoxic chemotherapy is the only treatment modality that produces rapid response. The frequency of response however depends on the agent, dose, and schedule. The response to therapy varies from 25% to over 50%. The most active agents include vinca alkaloids (vincristine, vinblastine), etoposide, anthracyclines and bleomycin. Combination therapies are more active than single agent treatments. However, the majority of cytotoxic agents cannot be administered for a prolonged period of time due to cumulative toxicity. Treatment with cytotoxic chemotherapy is palliative and the nearly all patients relapse within weeks of discontinuation of therapy.

Ovarian cancer can be separated into three major entities: epithelial carcinoma, germ cell tumors and stromal carcinomas. About 90% of the ovarian carcinomas are epithelial in origin, and the vast majority are diagnosed in postmenoposal women (Parker *et al.*, 1996). Epithelial cancer of the ovaries is usually detected only in advanced stages (III or IV) of the disease. The common pathway of tumor progression in ovarian carcinoma is via peritoneal dissemination, and the progressive accumulation of ascites is frequent with or without malignant tumor cells in the peritoneal fluid. It has been reported that ovarian carcinomas express VEGF mRNA and VEGF protein (Abu-Jaedeh *et al.*, 1996; Yamamoto S. *et al.*, 1997). VEGF is known to be produced by various solid tumors of epithelial origin and is thought to be involved in microvascular angiogenesis. In a recent study, Yamamoto and coworkers found that strong VEGF expression

plays an important role in the tumor progression of ovarian carcinoma (Yamamoto S. *et al.*, 1997).

Pancreatic carcinoma is the fifth leading cause of death from cancer. At the time of detection, pancreatic carcinoma has generally spread beyond curative surgery. Furthermore, other therapies such as radiation or chemotherapy have limited value. The vast majority of patients with pancreatic cancer die within 3-6 months following diagnosis. Thus other therapeutic strategies including inhibition of VEGF may be of value.

Malignant melanoma belongs to the few cancers whose incidence and mortality is increasing every year. Malignant melanoma can be considered as a disorder of cell differentiation and proliferation. Normal adult melanocytes originate from a precursor melanocyte that undergoes a series of differentiation events before reaching the final end cell differentiation state (Houghton *et al.*, 1982; Houghton *et al.*, 1987).

A number of growth factors such as EGF (Singletary *et al.*, 1987), NGF (Puma *et al.*, 1983), TGF (Derynk R *et al.*, 1987), PDGF (Westermark *et al*, 1986) and FGF (Moscateli *et al.*, 1986) have been shown to modulate the biology of melanoma *in vitro* and also are thought to have effects on tumor transformation and progression in the animal model. The clinical importance of these growth factors is as yet undetermined. VEGF and VEGF receptor expression have been detected on two melanoma cell lines (WW94 and SW1614) but data on human tumor tissue is not available.

4. SUMMARY OF THE INVENTION

These methods and compositions are needed for effective treatment of disorders characterized by abnormal proliferation of epithelial cells, including vascular endothelial cells. The present invention provides these and other advantages. The current invention discloses compositions of antisense oligonucleotides capable of inhibiting expression of VEGF. The antisense oligonucleotides of the invention inhibit growth of cultured cells that utilize VEGF as an autocrine growth factor. The antisense oligonucleotides of the invention block growth of cultured KS cells, cultured ovarian carcinoma cells, and cultured melanoma cells.

The invention also provides methods for treating Kaposi's sarcoma with inhibition of VEGF at therapeutic doses. Specifically, this invention demonstrates that KS can be lessened and that further tumor growth and spread can be blocked with the use of specific VEGF inhibitors, antisense oligonucleotides. This invention also details the parenteral administration of antisense VEGF inhibitors encapsulated in liposomes.

Other inhibitors of VEGF or VEGF receptors such as antibodies directed against VEGF, or against VEGF receptors (Flk-1/KDR, and Flt-1) or a soluble form of the receptor, which are exemplary but not exclusive, can be used for the treatment of KS. In particular, the invention provides compositions of the antisense oligonucleotides of the invention with such inhibitors.

Specific VEGF antisense oligonucleotides of the invention can also be used in a variety of diseases including cancers and precancerous conditions, proliferative retinopathy (diseases of the eye in which proliferation of the blood vessels cause visual loss), collagen vascular diseases, and skin diseases such as psoriasis and pemphigus. In one embodiment, the VEGF antisense oligonucleotides of the invention inhibit the growth of cultured ovarian carcinoma cells. In another embodiment the VEGF antisense oligonucleotides of the invention inhibit the proliferation of melanoma cells. In another embodiment, VEGF antisense oligonucleotides of the invention inhibit proliferation of pancreatic carcinoma cells.

This invention also discloses a method for discovering new inhibitors of VEGF using KS cell lines since the KS cell line produces and uses VEGF for its own growth. Inhibitors of VEGF also may be discovered by screening using ovarian, melanoma, or other cell lines that have autocrine VEGF growth activity (*i.e.* those cell lines sensitive to the VEGF inhibitors of the invention).

5. BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows that KS cells produce VEGF mRNA and protein at high levels when compared to other cell types such as fibroblasts, endothelial cells, and vascular smooth muscle cells. (A) Equal number of cells were used to extract total RNA, and Northern blot analysis were performed for VEGF. In addition the relative amount of RNA was assessed by probing the membranes for beta-actin, a house keeping gene. (B) Equal number of cells were grown in 25

cm^2 flasks and the supernatants were collected after 24 hr, and the VEGF levels were measured by ELISA.

Figure 2 illustrates expression of VEGF family members in KS and other tumor cell lines. VEGF expression is observed in KS cell lines, whereas no expression is observed in a B cell (23-2) and in a fibroblast cell line (T1). Expression . The RT-PCR product of VEGF members are seen on agarose gel. Kaposi's sarcoma cell line KSY-1 and cell line KS 6-3 express VEGF-A, VEGF-B, VEGF- C, VEGF- D, and placental growth factor (PIGF) in contrast to B lymphoma (23-2) and fibroblast (T1) cell lines that do not express these genes.

Figure 3 (A) shows that KS cells lines and primary KS tumors express both VEGF receptors (Flk-1/KDR and Flt -1). Several other cell lines including T-cell lines, B-cell lines and fibroblast cell lines were tested and none of which had any evidence of VEGF receptor expression. Normal human endothelial cells (HUVEC), as expected, served as positive controls. KS cells and control cells were grown in 75 cm^2 flasks until near confluence. Total cellular RNA was solubilized in guanidinium thiocyanate and cDNA synthesized. Using a specific primer pair for each of the two VEGF receptors, the mRNA transcripts were amplified and the products were resolved on agarose gel. (B) Integrity of the mRNA was confirmed by the demonstration of house keeping gene (β-actin) levels in the same cell lines.

Figure 4 demonstrates the expression of Flt 4 (VEGF-C receptor) in KS, other cell lines, and also the pair of samples of skin and KS lesions from the same patient. The figure shows RT-PCR product on agarose gel. Kaposi's sarcoma cell lines KSY-1, KS 6-3, express PIGF and Flt-4. In contrast B lymphoma (23-2) and fibroblast (T1) cell lines do not. Similarly, Flt-4 was expressed by the KS tumor lesion and not the skin from the same patient.

Figure 5 (A) shows that many of the tumor types, including colon (HT-29), breast (ZR-75), pancreas (panc), ovarian (ova-3), and melanoma (A-375), express VEGF-A and VEGF-C (Fig 5A), while expression of the other VEGF family members is heterogeneous (Figs. 5A and 5B).

Figure 6 shows that VEGF is an autocrine growth factor for KS tumor cells. Equal number of cells were plated and treated with different concentrations of AS-1/Veglin-1 (SEQ ID NO:1), AS-3/Veglin-3 (SEQ ID NO:2) or scrambled oligonucleotides (SEQ ID NO:30). The cell numbers represent the median of the experiments done in triplicates. (B) shows identical experiments done with several different cell types including KS cells (KSC-10, KS-59), human aortic smooth muscle cells (AoSM), human umbilical vein endothelial cells (HUVEC), fibroblast (T1), B lymphoma cells (23-1), T lymphoma cell line (HUT-78) using AS-1/Veglin-1 (SEQ ID NO:1), AS-3/Veglin-3 (SEQ ID NO:2), and scrambled oligonucleotides (SEQ ID NO:30). Figure 6E shows the effect of exogenous recombinant VEGF on HUVEC or KS cell proliferation. Recombinant VEGF (R&D Systems, Minneapolis, MN) was added to cells on day 1 and 3, and the cells were counted on day 5. The results represent the median of experiments done in triplicates. HUVEC showed dose dependent increase in cell proliferation while the response of KS cells was markedly blunted, possibly due to the occupancy of VEGF receptors by the endogenously produced ligand. Figure 6F shows the inhibition of endogenous VEGF production in KS cells by AS-1/Veglin-1 (SEQ ID NO:1) or AS-3/Veglin-3 (SEQ ID NO:2) makes cells sensitive to the exogenous VEGF. KS cells were treated with either SEQ ID NO: 1 or 2 alone at various concentrations or with SEQ ID NO:1 or 2 combined with VEGF. The results represent median of the experiments done in triplicates.

Figure 7 illustrates specificity of VEGF antisense oligonucleotides. KS cells were treated at various concentrations with either AS-1/Veglin-1 (SEQ ID NO:1) (A), AS-3/Veglin-3 (SEQ ID NO:2) (B), or scrambled oligonucleotide (SEQ ID NO: 30) (C). RT-PCR was done for VEGF mRNA (top) or β-actin (bottom). PCR products after various cycles of amplification (25-41) were resolved on agarose gel. Figure 7D reveals that AS-3/Veglin-3 (SEQ ID NO:2) but not scrambled oligonucleotides reduced the production of VEGF and the effect was dose dependent. Equal number of KS cells were plated in triplicate wells and treated with oligonucleotides. Supernatants were collected and assayed for VEGF levels by ELISA (R&D Systems, Minneapolis, MN). Figure 7E shows the cell proliferation assay with the oligonucleotides in two different ovarian carcinoma cell lines (both scrambled (SEQ ID NO:30) and antisense oligonucleotides AS-1 (SEQ ID NO:1) and AS-3 (SEQ ID NO:2). Both antisense oligonucleotides inhibited growth of ovarian carcinoma cell lines (Hey top panel, Hoc-7 bottom

panel), while scrambled oligonucleotides had no effect. Similar results were seen in Melanoma cell lines (Figure 7 F) 526 in the top panel and A375 in the bottom panel. These cell lines thus express VEGF receptors and use VEGF for autocrine growth activity.

Figure 8 shows that Veglin-1 (SEQ ID NO: 1) and Veglin-3 (SEQ ID NO: 2) are active *in vivo* to inhibit KS tumor growth. Immunodeficient mice bearing KS explants were treated with Veglin-1 (SEQ ID NO: 1) or Veglin-3 (SEQ ID NO: 2) or scrambled oligonucleotides, each given intraperitoneally daily for five days beginning one day after the tumor explants. The tumors were then allowed to grow for a total of 14 days. The tumor sizes were measured. The animals were then sacrificed and the tumors were removed and measured again.

Figure 9 illustrates the effects of liposomal encapsulation of Veglin-1 (SEQ ID NO: 1) and Veglin-3 (SEQ ID NO: 2). We have shown previously that liposomes deliver higher amounts of the drugs into the KS tumor cells than do free drugs. We thus encapsulated scrambled oligonucleotides and Veglin-3 (SEQ ID NO: 2) in the liposomes and treated the KS cells seeded at equal density in 24 well plates. The cell counts were performed on day 5 and the results are presented as the mean and ± SE of assays performed in triplicate. Liposomally encapsulated Veglin-3 (SEQ ID NO: 2) induced 50% inhibition of KS cell growth (IC_{50}) at doses 50 fold lower than required for free Veglin-3 (SEQ ID NO: 2).

Figure 10 shows that VEGF is a factor necessary for the survival of KS cells. Blocking VEGF production with Veglin-1 (SEQ ID NO: 1) or Veglin-3 (SEQ ID NO: 2) causes cell death in KS cell. KS cells were seeded at equal density in 75 cm^2 flasks, serum starved for 24 hr and treated with either Veglin-1 (SEQ ID NO: 1) or Veglin-3 (SEQ ID NO: 2) or scrambled oligonucleotide (SEQ ID NO:30), and the cell death was measured by examining the liberation of small DNA fragments (indicative of a specific method of cell death called programmed cell death or apoptosis). The DNA was extracted and size fractionated on the agarose gel.

Figure 11A illustrates the effect of Flk-1 and Flt-4 antibodies (separate and in combination) on KS Y1 cell proliferation. Flk-1 and Flt-4 antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. KS cells were plated at equal density and treated on day 1 and day 3 with various concentrations of the antibodies. Cell count was performed on day 5. The

results represent median of experiments done in triplicates. Figure 11B demonstrates that VEGF receptor antibodies (disruption of VEGF autocrine pathway) induce apoptosis of KS cells. KS cells were treated with various concentrations of VEGFR-2 (Flk-1) and VEGFR-3 (Flt-4) antibodies for 48 hours. The treated cells were incubated with fluorescein conjugated annexin V and propidium iodide for 15 minutes at room temperature in the dark and analyzed by flow cytometry. Cells undergoing apoptosis stained only with annexin V FITC reagent. The apoptotic cells show the shift of cell population to the right at X axis as shown above.

Figure 12 illustrates inhibition of KS tumor growth by anti-VEGFR2 (Flk-1) antibodies. KS Y-1 cells ($5x10^6$) cells were inoculated subcutaneously in lower back of Balb/C Nu+/Nu+ athymic mice. After 3 days of tumor growth, 200 ug of Flk-1 antibody was injected intraperitonealy daily for six consecutive days to one group of four mice, and the diluent alone to the control group of four mice. The tumor volume was measured twice a week for two weeks.

Figure 13 shows the effect of AS-3 (SEQ ID NO: 1) on human melanoma cells *in vivo*. Human melanoma cells were inoculated subcutaneously in lower back of Balb/C Nu+/Nu+ athymic mice. Tumor size was measured for control animals receiving a scrambled oligonucleotide (SEQ ID NO: 30) or antisense oligonucleotide (SEQ ID NO: 2).

Figure 14 shows the position of selected antisense oligonucleotides denoted by asterisks in Table 1 relative to the gene sequence for VEGF-A. Asterisks correspond to those listed in Table 1. Individual SEQ ID NOS are to the left of the brackets. Numbers to the right of the brackets represent the VEGF-165 isoform sequences that the antisense molecules are complementary to. Gene sequence numbers are according to Leung *et al.*, (1989) where numbering started at the translation start site. The sequences of VEGF-A, -C, and –D are aligned, with 3/3 matches indicated by bold faced type, and 2/3 matches by underlining.

6. DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

The term "response" means a halt in the progression of KS lesions and/or a decrease in tumor size without accompanying unwanted side effects.

The term "partial response" means a complete flattening of more than 50% of the raised lesions lasting for four weeks or more.

The term "pharmacologically acceptable carrier" means any chemical approved for use as part of a drug formulation by a regulation agency of the federal or state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans.

The term "therapeutically effective dose" of a VEGF antagonist means an amount calculated to achieve and maintain a therapeutically effective level in the tumor, if applied to the tumor, or in the plasma, if administered systematically, as to substantially inhibit the proliferation of KS cells. It is preferred that the therapeutic amount be sufficient to inhibit proliferation of more than 50 percent of KS cells *in vitro*. Of course, the therapeutic dose will vary with the potency of each VEGF antagonist in inhibiting KS cell growth *in vitro*, and the rate of elimination or metabolism of the VEGF antagonist by the body in the tumor tissue and /or in the plasma.

The term "IC_{50}" means the concentration of a substance that is sufficient to inhibit a test parameter (such as, e.g., cell growth, tumor volume, VEGF protein expression, etc.) by about 50 percent.

The term "antagonist" means a compound that prevents the synthesis of the target molecule or binds to the cellular receptor of the target molecules or an agent that blocks the function of the target molecule.

The term "antisense oligonucleotides" means a sequence of nucleic acids constructed so as to bind to the mRNA encoding a certain protein and thereby prevent translation of the mRNA into protein.

The terms "sense oligonucleotides" "oligonucleotide fragment" or "polynucleotide fragment" "portion," or "segment" refer to a stretch of nucleotide residues which is long enough to use in PCR or various hybridization procedures to identify or amplify identical or related parts of mRNA or DNA molecules. The polynucleotide compositions of this invention include RNA, cDNA, genomic DNA, synthetic forms, and mixed polymers, both sense and antisense strands, and may be chemically or biochemically modified or may contain non-natural or derivatized nucleotide bases, as will be readily appreciated by those skilled in the art. Such modifications include, for example, labels, methylation, substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as uncharged linkages (*e.g.*, methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, *etc.*), charged linkages (*e.g.*, phosphorothioates, phosphorodithioates, *etc.*), pendent moieties (*e.g.*, polypeptides), intercalators (*e.g.*, acridine, psoralen, *etc.*), chelators, alkylators, and modified linkages (*e.g.*, alpha anomeric nucleic acids, *etc.*) Also included are synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence via hydrogen bonding and other chemical interactions. Such molecules are known in the art and include, for example, those in which peptide linkages substitute for phosphate linkages in the backbone of the molecule.

The term "scrambled oligonucleotide" means a sequence of nucleic acid constructed so as to match the nucleic acids content but not the sequence of a specific oligonucleotide.

ROLE OF VEGF IN KAPOSI'S SARCOMA, OVARIAN CARCINOMA AND MELANOMA.

In vitro studies have shown that KS cells express VEGF at high levels. In addition, VEGF receptors (Flt-1 and KDR) were shown to be expressed in KS cell lines. Furthermore, the addition of VEGF to the KS cells was shown to enhance KS cell growth, although it was less dramatic than seen in endothelial cells. These findings for the first time showed that KS cells express functional VEGF receptors and that VEGF acts as a growth factor for KS. This is the first demonstration of any tumor cell type to use VEGF for its own growth. The role of VEGF was documented after the VEGF expression was blocked in KS cells with the use of novel antisense oligonucleotides (Veglin-1 (SEQ ID NO: 1) and Veglin-3 (SEQ ID NO: 2)). These

findings indicated that under the normal conditions, the VEGF produced by the tumor cells binds with the VEGF receptors and keeps the cells proliferating. In addition, it was shown that the blockage of VEGF production by the novel antisense oligonucleotides (e.g., SEQ ID NOS: 1 and 2) lead to KS cell death, indicating that VEGF not only is required for the growth of the tumor cells, but also for KS cell survival. These findings were then confirmed in the primary tumor tissues showing that VEGF and VEGF receptors are expressed in the tumor, while the normal adjoining tissue biopsies did not show expression of either VEGF or VEGF receptors.

MOUSE MODEL SYSTEM

The relevance of the cell culture findings to the ability of the antisense oligonucleotides of the invention to be used for the treatment of Kaposi's Sarcoma were confirmed by performing experiments *in vivo* in a mouse model of KS. KS tumors implanted in immunodeficient mice were treated only for a short period and the growth of the tumor was studied for several additional days. Novel antisense oligonucleotides (SEQ ID NO: 1 and SEQ ID NO: 2) blocked the growth of the tumor *in vivo*.

ANTISENSE OLIGONUCLEOTIDES

As described herein, the present invention provides a number of oligonucleotide sequences that specifically inhibit the synthesis of VEGF protein and thus are able to block KS tumor growth. In a preferred embodiment these oligonucleotides include Veglin-1 (AS-1) which has the following sequence SEQ ID NO: 1: 5' -AGA CAG CAG AAA GTT CAT GGT-3' and Veglin-3 (AS-3) which has the following sequence SEQ ID NO: 2: 5'-TGG CTT GAA GAT GTA CTC GAT-3'. In another preferred embodiment, the antisense oligonucleotides of the invention have sequences SEQ ID NOS: 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 21, 28 and 29. Antisense oligonucleotides can also comprise truncated fragments of such sequences.

With the published nucleic acid sequences of the target VEGF polynucleotides and this disclosure provided, those of skill in the art will be able to identify, without undue experimentation, other antisense nucleic acid sequences that inhibit VEGF expression. For example, other sequences targeted specifically to human VEGF nucleic acid can be selected based

on their ability to be cleaved by RNAse H, or to displace the binding of the disclosed antisense oligonucleotides from a nucleic acid encoding VEGF or a portion thereof.

In one embodiment, antisense oligonucleotides are chosen such that they are able to inhibit the growth of cultured Kaposi's sarcoma cells. In a preferred embodiment, these oligonucleotides are able to inhibit the proliferation of the Kaposi's sarcoma cells at IC_{50} concentrations of less than about 1.5 micromolar (uM). A particularly preferred technique for determining the concentration antisense oligonucleotide capable of inhibiting proliferation of a Kaposi's sarcoma cell line is the method outlined in Examples 3 and 9 using KS cells.

Effective concentrations of antisense oligonucleotides can be determined by techniques other than inhibition of cultured Kaposi's sarcoma cells. Such assays can be calibrated to correspond to the data provided, for example, in Table 1. Another suitable assay that can be used is the determination of the effect of the antisense oligonucleotide on mRNA levels in a cell, such as described in Example 10. In one embodiment, antisense oligonucleotides are capable of reducing mRNA levels for one or more forms of VEGF by a factor of about 1.5 or more. In another embodiment, the antisense oligonucleotide is capable of reducing the mRNA levels of 2 or more forms of VEGF by a factor of 2 or more.

The oligonucleotides of the invention are composed of ribonucleotides, deoxyribonucleotides, or a combination of both, with the 5' end of one nucleotide and the 3' end of another nucleotide being covalently linked. These oligonucleotides are at least 14 nucleotides in length, but are preferably 15 to 28 nucleotides long, with 15- to 25-mers being the most common.

These oligonucleotides can be prepared by the art recognized methods such as phosphoramidite or H-phosphonate chemistry which can be carried out manually or by an automated synthesizer as described in Uhlmann *et al.* (Chem. Rev. (1990) 90:534-583).

The oligonucleotides of the invention may also be modified in a number of ways without compromising their ability to hybridize to VEGF mRNA. For example, the oligonucleotides may contain other than phosphodiester internucleotide linkages between the 5' end of one nucleotide

and the 3' end of another nucleotide in which the 5' nucleotide phosphodiester linkage has been replaced with any number of chemical groups. Examples of such chemical groups include alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, phosphoramidates, phosphate esters, carbamates, acetamidate, carboxymethyl esters, carbonates, and phosphate triesters. Oligonucleotides with these linkages can be prepared according to known methods (see, *e.g.*, Uhlmann *et al.* (1990) Chem. Rev. 90:543-583). The term oligonucleotides also encompasses heterpolymers with totally distinct backbone structures such as polyamide nucleic acids (Nielsen, P.E. (1999). Curr. Opin. Struct. Biol. 9:353-7.)

The preparation of these and other modified oligonucleotides is well known in the art (reviewed in Agrawal *et al.* (1992) Trends Biotechnol. 10:152-158). For example, nucleotides can be covalently linked using art-recognized techniques such as phosphoramidate, H-phosphonate chemistry, or methylphosphoramidate chemistry (*see*, *e.g.*, Uhlmann *et al.* (1990) Chem. Rev. 90:543-584; Agrawal *et al.* (1987) Tetrahedron Lett. 28:(31):3539-3542); Caruthers *et al.* (1987) Meth. Enzymol, 154:287-313; U.S. Pat. No. 5,149,798). Oligomeric phosphorothioate analogs can be prepared using methods well known in the field such as methoxyphosphoramidite (*see*, *e.g.*, Agrawal *et al.* (1988) Proc. Natl. Acad. Sci. (USA) 85:7079-7083) or H-phosphonate (*see*, *e.g.*, Froehler (1986) Tetrahedron Lett. 27:5575-5578) chemistry. The synthetic methods described by Bergot *et al.* (J. Chromatog. (1992) 559:35-42) can also be used. Oligonucleotides of the invention may also have modified sugars, including pendant moieties on the 2' position, and modified nucleobases, including propynyl modified bases, as well as other nonnatural bases with suitable specificity.

ANTIBODIES

The present invention also provides polyclonal and/or monoclonal antibodies, including fragments and immunologic binding equivalents thereof, which are capable of specifically binding to the polynucleotide sequences of the specified gene and fragments thereof, as well as the corresponding gene products and fragments thereof. In general, techniques for preparing polyclonal and monoclonal antibodies as well as hybridomas capable of producing the desired

antibody are well known in the art (Campbell, 1984; Kohler and Milstein, 1975). These include, *e.g.*, the trioma technique and the human B-cell hybridoma technique (Kozbor, 1983; Cole, 1985).

Any animal (mouse, rabbit, *etc.*) that is known to produce antibodies can be immunized with the immunogenic composition. Methods for immunization are well known in the art and include subcutaneous or intraperitoneal injection of the immunogen. One skilled in the art will recognize that the amount of the protein encoded by the nucleic acids of the present invention used for immunization will vary based on the animal which is immunized, the antigenicity of the immunogen, and the site of injection. The protein which is used as an immunogen may be modified or administered in an adjuvant to increase its antigenicity. Methods of increasing antigenicity are well known in the art and include, but are not limited to, coupling the antigen with a heterologous protein (such as globulin, β-galactosidase, KLH, etc.) or through the inclusion of an adjuvant during immunization.

For monoclonal antibodies, spleen cells from the immunized animals are removed, fused with myeloma cells, such as SP2/0-Ag14 myeloma cells, and allowed to become monoclonal antibody producing hybridoma cells. Any one of a number of methods well known in the art can be used to identify hybridoma cells that produce an antibody with the desired characteristics. These include screening the hybridomas with an enzyme-linked immunosorbent assay (ELISA), western blot analysis, or radioimmunoassay (RIA) (Lutz, 1988). Hybridomas secreting the desired antibodies are cloned and the immunoglobulin class and subclass may be determined using procedures known in the art (Campbell, 1984).

Techniques described for the production of single chain antibodies (U.S. Patent No. 4,946,778) can be adapted to produce single chain antibodies to the proteins of the present invention. For polyclonal antibodies, antibody-containing antisera is isolated from an immunized animal and is screened for the presence of antibodies with the desired specificity using one of the above described procedures.

In the present invention, the above-described antibodies are used in a labeled form to permit detection. Antibodies can be labeled, *e.g.*, through the use of radioisotopes, affinity labels (such as biotin, avidin, *etc.*), enzymatic labels (such as horseradish peroxidase, alkaline

phosphatase, *etc.*) fluorescent labels (such as fluorescein or rhodamine, *etc.*), paramagnetic atoms, etc. Procedures for accomplishing such labeling are well-known in the art, *e.g.*, *see* Sternberger, 1970; Bayer, 1979; Engval, 1972; Goding, 1976. The labeled antibodies of the present invention can then be used for *in vitro*, *in vivo*, and *in situ* assays to identify the cells or tissues in which a fragment of the polypeptide of interest is expressed. Preferred immunoassays are the various types of ELISAs and RIAs known in the art (Garvey, 1977). The antibodies themselves also may be used directly in therapies or as diagnostic reagents.

PHARMACEUTICAL COMPOSITIONS

The synthetic oligonucleotides of the invention may be used as part of a pharmaceutical composition when combined with a physiologically and/or pharmaceutically acceptable carrier. The characteristics of the carrier will depend on the route of administration. Such a composition may contain, in addition to the synthetic oligonucleotide and carrier, diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials well known in the art.

The pharmaceutical composition of the invention may also contain other active factors and/or agents which enhance inhibition of VEGF expression or which will reduce neovascularization. For example, combinations of synthetic oligonucleotides, each of which is directed to different regions of the VEGF mRNA, may be used in the pharmaceutical compositions of the invention. The pharmaceutical composition of the invention may further contain nucleotide analogs such as azidothymidine, dideoxycytidine, dideosyinosine, and the like. Such additional factors and/or agents may be included in the pharmaceutical composition to produce a synergistic effect with the synthetic oligonucleotide of the invention, or to minimize side-effects caused by the synthetic oligonucleotide of the invention. Conversely, the synthetic oligonucleotide of the invention may be included in formulations of a particular anti-VEGF or anti-neovascularization factor and/or agent to minimize side effects of the anti-VEGF factor and/or agent.

The pharmaceutical composition of the invention may be in the form of liposomes in which the synthetic oligonucleotides of the invention is combined, in addition to other pharmaceutically acceptable carriers, with amphipathic agents such as lipids which exist in aggregated form as

micelles, insoluble monolayers, liquid crystals, or lamellar layers which are in aqueous solution. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. One particularly useful lipid carrier is lipofectin. Preparation of such liposomal formulations is within the level of skill in the art, as disclosed, for example, in Szoka *et al.*, Ann. Rev. Biophys. Bioeng. 9:467 (1980), U.S. Pat. Nos. 4,235,871, 4,501,728, 4,837,028; the text Liposomes, Marc J. Ostro, ed., Chapter 1, Marcel Dekker, Inc., New York (1983), and Hope *et al.*, Chem. Phys. Lip. 40:89 (1986), all of which are incorporated herein by reference.

The pharmaceutical composition of the invention may further include compounds such as cyclodextrins and the like which enhance delivery of oligonucleotides into cells, as described by Zhao *et al.* (Zhao Q, Temsamani J, Agrawal S (1995) Use of cyclodextrin and its derivatives as carriers for oligonucleotide delivery. *Antisense Res. Dev.* 5(3):185-92), or slow release polymers.

The following examples illustrate the manner in which the invention can be practiced. It is understood, however, that the examples are for the purpose of illustration and the invention is not to be regarded as limited to any of the specific materials or conditions therein.

EXAMPLES

Materials and Methods

Antibodies used include p-130 and Tie-1 antibodies. Antibody p130 is an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 1120 - 1139 mapping at the carboxy terminus of p130 of human origin. Antibody Tie-1 is an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 1121 - 1138 mapping at the carboxy terminus of the precursor form of Tie-1 of human origin.

Isolation of KS cells. AIDS-KS-derived spindle cell strains were isolated from primary tumor tissues as described previously (Nakamura *et al.* 1988). Cells were cultured continuously in 75 cm^2 flasks coated with 1.5% gelatin, in KS medium consisting of the following: RPMI 1640 (Life Technologies),100 U/mL penicillin, 100 ug/mL streptomycin, 2 mM glutamine, essential and nonessential amino acids, 10% fetal bovine serum (FBS, Life Technologies), and 1% Nutridoma-

HU (Boehringer Mannheim). The primary isolates were characterized to determine their phenotype using an immunofluorescent assay. The markers expressed include endothelial cell markers; UEA-1 binding sites, EN-4, PALE; smooth muscle cell specific markers including vascular smooth muscle cell specific alpha actin; macrophage specific marker including CD14. Neoplastic cell line KSY-1 is propagated similarly and has a similar phenotype.

Example 1

Expression of VEGF- and VEGF-C receptors (flt-4) by KS cells

In vitro studies showed that KS cells express all members of the VEGF family at high levels. *Flt-1* and *KDR* mRNA expression was assayed in KS cell line (KSY1), HUVEC, normal skin and KS tumor tissue from an HIV+ patient, T1 (fibroblast), 23-1 (B-lymphoma) and HUT-78 (T cell lymphoma). Equal amounts of RNA were reverse transcribed to generate cDNA. cDNAs were subjected to Flt-1 and KDR specific PCR amplifications (500 and 700 bp products respectively) (Fig. 3A) using paired primers, or as a control, cDNAs from all samples were subjected to β-actin specific PCR amplification (548 bp product)(Fig. 3B). VEGF-C receptor (flt-4) expression was examined in a similar manner (Fig. 4).

Example 2

Expression of VEGF mRNA and production of VEGF protein by KS cells

VEGF mRNA expression was analyzed in several AIDS-KS cell lines. Preferably, 15 ug of total RNA from KSC10, KSC29, KSC13, KSC59 and KSY1, KSC10, HUVEC and AoSM (Fig. 1A) were electrophoresed, blotted and hybridized to the human VEGF cDNA (Fig. 1A top) and β-actin probe (Fig. 1A bottom). Supernatants from equal numbers of cells from KSY1, KSC10, AoSM, HUVEC and T1 were collected after 48 hours-and analyzed for VEGF protein by ELISA (Fig. 1B).

Example 3

Effect of VEGF antisense oligonucleotides on KS cell growth

KS cells were treated with VEGF antisense AS-1 (Veglin-1; SEQ. ID NO: 1), AS-3 (Veglin-3; SEQ. ID NO: 2), and the scrambled oligonucleotide at concentrations ranging from 1

to 10 μM. The scrambled oligonucleotide used in these and subsequent experiments has the following sequence: (SEQ ID NO: 33) 5'- TAC GTA GTA TGG TGT ACG ATC -3'. Cell proliferation was measured on day 3 (Fig. 6A). Data represent the mean ± standard error of assays performed in triplicate. Figure 6E demonstrates the effect of rhVEGF on the growth of KS and HUVEC cells. Cells were seeded at 1 X 10^4 cells per well in 24 plates and treated with rhVEGF (1 to 10 ng/mL) for 48 hours. Cell counts were performed and the results represent the mean ± SD of an experiment performed in quadruplicate (Fig. 6E). rhVEGF abrogates the effect of VEGF antisense on AIDS-KS cell growth. KS cells were seeded at a density of 1 x 10^4 cells per well in 24 well plates. Cells were treated with 1 and 10 μM of AS-3 (Veglin-3) alone or with rhVEGF(10 ng/mL) on day 1 and day 2. Cell proliferation was measured after 72 hours. The data (Fig. 6F) represent the mean ± standard deviation of two experiments performed in quadruplicate. As shown by the results summarized in Figure 6, incubation of AIDS-KS cells for 3 days with antisense oligonucleotides results in a dose dependent inhibition of KS cell growth, as measured by cell count. In contrast, the sense oligonucleotides did not result in significant inhibition of KS cell growth. These findings indicate that VEGF is an autocrine growth factor for KS cells.

Example 4

Specificity of VEGF antisense oligonucleotides

Antisense oligonucleotides to various coding regions of the human VEGF gene were synthesized and phosphorothioate modified to reduce degradation. Equal number of cells were seeded in 24 well plates. The molar concentration-dependent potency of VEGF antisense oligonucleotides for inhibition of growth of KS cells (KSC-10, KSC –59) was examined in the cell proliferation assays after exposure of the cells on day 1 and 2, and cell counts performed on day 3. Viable cell counts were determined by Coulter counter. Each value is the mean + SE of assays performed in triplicate. The controls included scrambled phosphorothioate modified oligonucleotides. In addition, the control experiments included cell lines including T-cell lines (HUT-78), B-cell lines (23-1), smooth muscle cells (AoSM), endothelial cells (HUVEC) and fibroblast (T1). Two antisense oligonucleotides tested in this experiment showed inhibition of KS cell lines, while several others had no significant effect. These oligonucleotides AS-1 and AS-3

also are referred to as SEQ ID NO: 1 and SEQ ID NO: 2. It is also notable that SEQ ID NO: 1 and SEQ ID NO: 2 had no significant effect on the growth of various control cell lines, such as B cell lines, T cell lines and fibroblast cell lines.

Cells were seeded at equal density and treated with Veglin-1 (SEQ ID NO:1) or Veglin-3 (SEQ ID NO:2), or scrambled oligonucleotides (at 0, 1, 5 & 10 μM), followed by a cell count (Figs. 6B, 6C and 6D) and extraction of total cellular RNA. Total RNA was isolated from AIDS-KS cells treated with various concentrations of AS-1/Veglin-1 (SEQ ID NO: 1) (Fig. 7A), AS-3/Veglin-3 (SEQ ID NO: 2) (Fig. 7B) and scrambled oligonucleotide (SEQ ID NO: 33) (Fig 7C). Total RNA was reverse transcribed to generate cDNA. PCR was carried out for VEGF and β-actin. Upper panel shows PCR products of 535 and 403 bp corresponding to VEGF,2S and VEGF,6S mRNA species of VEGF. Lower panels show the 548 bp PCR product of β-actin. NT= No treatment; M= Molecular size marker, 25-41 and 18-33 represent the number of PCR cycles. The results demonstrate that AS-1/Veglin-1 and AS-3/Veglin-3 specifically reduce the accumulation of VEGF,2S and VEGF,6S mRNA species in a dose-dependent manner. Figure 7D illustrates that these VEGF oligonucleotides inhibit the production of VEGF protein in KS cells. The supernatants of KS cells treated with AS3 (Veglin-3) and scrambled VEGF antisense oligonucleotide were collected at 48 hr and VEGF protein was quantitated by ELISA. The results represent the mean ± standard deviation of two separate experiments done in duplicate.

Example 5

Inhibition of tumor growth by VEGF oligonucleotides

VEGF antisense oligonucleotide effects on tumor growth were studied in nude mice. KS-Y1 cells (1×10^7) were inoculated subcutaneously in the lower back of Balb/C/Nu+/NU+ athymic mice. AS-1/Veglin-1 (SEQ ID NO:1), AS-3/Veglin-3 (SEQ ID NO:2), Scrambled (S) (SEQ ID NO: 33) VEGF oligonucleotides and diluent (PBS) were injected intra-peritoneally daily for five days (day 2 to 6). Mice were sacrificed on day 14 and tumor size was measured. Data represent the mean ± standard deviation of 10 mice in each group. Figure 8 illustrates the drastic reduction in tumor growth as a result of treatment with AS-1 (SEQ ID NO: 1) or AS-3 (SEQ ID NO: 2). Similar experiments done on human melanoma tumor cells (M21) implanted in mice

show marked reduction in tumor growth (Fig. 13). Experiments using human pancreatic carcinoma cell lines implanted in the pancreas of mice also showed tumor reduction, decrease in tumor spread, ascites and improved survival. In addition the serum and ascites VEGF levels were reduced to normal levels with AS-3.

Example 6

Liposomal encapsulation of VEGF antisense oligonucleotides

KS cells were treated with oligonucleotides encapsulated in neutral liposomes at various concentrations on day 1 and day 2 and the cell count was performed on day 3. Cell proliferation was measured 72 hours after start of treatment. The data represent the mean ± standard deviation of two experiments performed in quadruplicate. Liposomal encapsulation increased the apparent potency of the VEGF antisense oligonucleotides. Over 50% reduction in the cell growth was observed at concentration 50 fold below that required for free oligonucleotides (*cf.* Fig. 6F, Fig. 9, bottom panel) Furthermore scrambled oligonucleotides at the same concentrations had no inhibitory effects (Fig. 9, top panel).

Example 7

Effect of VEGF on KS cell survival

In addition, the effect of antisense oligonucleotides (AS-3) on KS cell survival was studied. KS cells were treated with various concentrations of oligonucleotides. The DNA was extracted and separated on agarose gel. As illustrated in Figure 10 antisense oligonucleotides at concentrations of 1 uM and above showed evidence of cell death through the mechanism of programmed cell death, also called apoptosis (Fig. 10 left panel). Scrambled oligonucleotides (SEQ ID NO:30) had no effect at concentrations of up to 10 uM (Fig. 10 right panel). This example shows that VEGF is not only an autocrine growth factor for KS cells, but is also necessary for cell survival.

Example 8

Effect of Flk-1/KDR and Flt-4 antibodies on KS cell growth

Figure 11A illustrates that Flk-1/KDR and Flt-4 antibodies inhibit KS cell growth in a dose-dependent manner. A synergistic effect was observed when they are administered in

combination. A similar effect was observed on the receptors, *i.e.* antibodies to Flk-1 and Flt-1 induced apoptosis in a dose-dependent manner, with an additive effect when both were combined (Fig. 11B). In contrast, antibodies to another endothelial cell receptor tyrosine kinase which also is expressed on KS cells had no effect. The *in vivo* activity of VEGF receptor (Flk-1) has been shown in vivo. Relative to the controls, Flk-1 antibody treated mice bearing KS tumor had markedly reduced tumor growth (Fig. 12).

EXAMPLE 9

Use of Antisense Oligonucleotides to Inhibit Cultured KS, Ovarian Carcinoma and Melanoma Cells

Cell Proliferation Assay

The immortalized KS cell lines KS Y-1 and KS-SLK, were grown in wells coated with 1.5% gelatin in KS medium consisting of RPMI-1640 (Life Technologies, Gaithersburg MD), 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, essential and non-essential amino acids, 10% fetal bovine serum (FBS: Life Technologies), and 1% Nutridoma-HU (Boehringer Mannheim, Indianapolis IN). The Kaposi's Sarcoma cell line KS Y-1 is available from ATCC (CRL-11448) and is the subject of US patent 5,569,602. The Kaposi's sarcoma cell line KS-SLK is available from Dr. E. Rubinstein, Chaim Sheba Medical Center, Tel-Hashomer, Israel. Human umbilical vein epithelial cells (HUVEC) (Clonetics, San Diego CA) were grown in medium containing epidermal growth factor and according to the instructions of the supplier. T1 fibroblasts; ovarian carcinoma Hoc-7 and Hey; human melanoma A375, 397 and 526 cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS and antibiotics as above. The ovarian carcinoma cell lines Hoc-7 and Hey were obtained from Dr. Donald Buick, University of Toronto, Canada. The melanoma cell line A375 was obtained from the American Type Culture Collection (ATCC number CRL-1619). The melanoma cell lines 397 and 523 were obtained from Dr. Steven Rosenberg, Surgery Branch, Division of Cancer Treatment; National Cancer Institute, National Institutes of Health, Bethesda, MD. All cells were seeded at a density of 1.0×10^4 cells/well in 24-well plates in appropriate growth medium on day 0. After allowing the cells to attach overnight, cells were treated with varying concentrations (1 to 10 μM) of the VEGF antisense oligonucleotide on days 1 and 3. On day 5 cell growth was assayed using 3-[4,5-

dimethylthiazol-1-yl]-2,5-diphenyltetrazolium bromide (MTT). Wells were treated with 0.5 mg/ml MTT in 90% isopropanol, 0.5% SDS and 40 mM HCl. Developed color was read at 490 nm in an ELISA plate reader (Molecular Devices, CA) using isopropanol as a blank.

Antisense oligonucleotides corresponding to regions of VEGF mRNA were synthesized by standard chemical techniques. The oligonucleotides were synthesized as phosphorothioate without further modification. IC_{50} values were determined using the cell proliferation assay as described above and are reported in Table 1.

Table 1. Activity of VEGF antisense oligonucleotides in Kaposi's sarcoma (KS), Ovarian carcinoma (OV) and melanoma (MEL)

	SEQ ID NO:	SEQUENCE	Coding sequence position	IC_{50} KS (μM)	IC_{50} OV (μM)	IC_{50} MEL (μM)
	3	ATTGCAGCAG CCCCCACATC G	320-299	4.8	10	6.7
	4	GCAGCCCCCA CATCGGATCA G	314-293	2.8	7.6	3.8
	5	CCCACATCGG ATCAGGGGCA C	308-287	10	>10	>10
	6	TCGGATCAGG GGCACACAGG A	302-281	10	>10	>10
	7	CAGGGGCACA CAGGATGGCT T	296-275	>10	>10	>10
	8	CACACAGGAT GGCTTGAAGA T	290-270	8.2	>10	>10
*	9	ACACAGGATG GCTTGAAGAT G	289-269	0.85	1.6	1.6
*	10	CACAGGATGG CTTGAAGATG T	288-268	0.9	1.9	1.5
*	11	ACAGGATGGC TTGAAGATGT A	287-267	1.6	3.4	2.7
*	12	CAGGATGGCT TGGAGATGTA C	286-266	0.9	1.8	0.9
**	13	AGGATGGCTT GGAGATGTAC T	285-265	0.4	1.1	0.6
**	14	GGATGGCTTG AAGATGTACT C	284-264	0.38	1.1	0.7
*	15	GATGGCTTGA AGATGTACTC G	283-263	1.11	2.4	1.2
*	16	ATGGCTTGAA GATGTACTCG A	282-262	1.42	3.0	2.5
*	2	TGGCTTGAAG ATGTACTCGA T	281-261	2.1	5.2	3.2
**	17	GGCTTGAAGA TGTACTCGAT C	280-260	0.5	1.2	0.5
*	18	GCTTGAAGAT GTACTCGATC T	279-259	1.38	3.1	2.2
	19	CTTGAAGATG TACTCGATCT C	278-258	2.42	6.0	3.7
*	20	GGATGGCTTG AAGATGTACT	284-265	0.95	2.7	1.0
*	21	GGATGGCTTG AAGATGTAC	284-266	1.1	2.8	1.4
	22	GGATGGCTTG AAGATGTA	284-267	3.8	>10	5.8
	23	GGCTTGAAGA TGTACTCGAT	280-261	4.8	>10	7.1
	24	GCTTGAAGAT GTACTCGAT	279-261	4.6	>10	6.2
	25	CTTGAAGATG TACTCGAT	278-261	6.2	>10	8.6
	26	TGGCTTGAA GATGTACTCG A	281-262	3.4	>10	4.7
	27	TGGCTTGAAG ATGTACTCG	281-263	6.9	>10	>10
	28	GGGCACACAG GATGGCTTGA AGATGTACTC GAT	293-261	0.6	1.2	1.3
*	29	GGGCACACAG GATGGCTTGA AGA	293-271	0.7	1.5	1.2

Nucleotide numbering shown in the fourth column is from the translation start site of VEGF-165 isoform as published in: Leung DW, Cachianes G, Kuang W-J, Goeddel DV, and Ferrara N. (1989) "Vascular endothelial growth factor is a secreted angiogenic mitogen." *Science* **246**:1306-1309. The antisense molecules are represented, as per the convention, in the 5'→ 3' orientation. Antisense molecules are complements to the coding strand of the DNA, which also by convention is represented and numbered 5'→ 3'. Nucleic acids anneal to strands with opposing polarity, therefore the numbers in the fourth column, which represent the gene sequence appear 3'→5' (higher to lower). IC_{50} values indicate the concentration of antisense oligonucleotide necessary to inhibit cell proliferation by 50%.

EXAMPLE 10

Effect of Antisense Oligonucleotides on Expression of VEGF-A, -C and -D

KS Y-1 cells were seeded at a density of 1 x 10^4 per well in gelatin-coated plates on day 0. The cells then were treated individually with antisense oligonucleotides SEQ ID NOS: 3-29, at various concentrations (0, 1, 5, and 10 uM) on day 1. Cells were harvested and total RNA was extracted on day 3. cDNAs were synthesized by reverse transcriptase using a random hexamer primer in a total volume of 20 ul (Superscript, Life Technologies Inc.). Five microliters of the cDNA reaction were used for PCR using gene-specific primers for i)VEGF-A, ii) VEGF-C and iii) VEGF-D. Each PCR cycle consisted of denaturation at 94 °C for 1 min, primer annealing at 60 °C for 2 min, and extension at 72 °C for 3 min. The samples were amplified for 41 cycles, and 5 ul aliquots were removed from the PCR mixtures after every 4 cycles starting at cycle 25. Amplified product was visualized on a 1.5 % agarose gel containing ethidium bromide. All samples analyzed for VEGF-A, -C or -D expression also were analyzed for β-actin expression to confirm the integrity and quantity of the RNA. Table 2 shows the effect of antisense oligonucleotides SEQ ID NO:2 and SEQ ID NO:14 on the expression of various VEGF members corrected for beta-actin amplification.

Table 2. Quantitation of mRNA levels in response to antisense oligonucleotides.

Table 2 demonstrates the effects of various antisense oligonucleotides on the expression of VEGF protein family members. AS-3/Veglin-3 (SEQ ID NO: 2) produced a dose-dependent decline in VEGA-A mRNA levels. AS-3/Veglin-3 had no significant effect on VEGF-C, VEGF-D or PIGF expression. In contrast, SEQ ID No: 14 produced dose-dependent declines in the mRNA levels of VEGF-A, -C, and -D. This antisense molecule lowered VEGF-A mRNA

levels from 2.7-3 fold at 1 uM and 4.6-6.3 fold at 5 uM. Furthermore the levels of VEGF- C and VEGF-D declined to similar magnitude and were 3-fold reduced at 1 uM and 6-fold reduced at 5 uM concentrations. There was no significant effect on PIGF. Neither of these oligonucleotides produced a decline in mRNA levels of beta-actin, a house keeping gene.

Table 2. Quantitation of mRNA levels in response to antisense oligonucleotides.

	Fold Decline in mRNA levels				
	VEGF-A	VEGF-C	VEGF-D	PIGF	β-actin
AS-3/Veglin-3/SEQ ID NO:2					
1 uM	1.6	none	none	none	none
5 uM	3.2	none	none	none	none
SEQ ID NO: 14					
1 uM	2.7-3.0	3	3	none	none
5 uM	4.6-3.2	6	6	none	none

The ability of an antisense oligonucleotide to inhibit cell growth may be dependent on its ability to inhibit multiple forms of VEGF. Table 3 shows the relative effects of antisense oligonucleotides directed towards VEGF on VEGF,-A, -C, and -D gene expression. Particular, high affinity sequences are capable of inhibiting multiple forms of VEGF. Those antagonists showing the largest inhibition are marked with two asterisks. Other antagonists showing broad activity against multiple forms of VEGF are marked with a single asterisk. Using these data, one of skill in the art can select an appropriate oligonucleotide sequence for inhibiting a specific form of VEGF, or for inhibiting growth of tumor cells, a sequence that broadly inhibits multiple VEGF forms.

Table 3. Effect of antisense oligonucleotides on VEGF-A, -C and –D gene expression.

	SEQ ID NO:	SEQUENCE	VEGFA	VEGFC	VEGF D
	3	ATTGCAGCAG CCCCCACATC G	–	–	–
	4	GCAGCCCCCA CATCGGATCA G	–	–	–
	5	CCCACATCGG ATCAGGGGCA C	–	–	–
	6	TCGGATCAGG GGCACACAGG A	–	–	–
	7	CAGGGGCACA CAGGATGGCT T	–	–	–
	8	CACACAGGAT GGCTTGAAGA T	–	–	–
*	9	ACACAGGATG GCTTGAAGAT G	+	+	+
*	10	CACAGGATGG CTTGAAGATG T	+	+	+
*	11	ACAGGATGGC TTGAAGATGT A	+/-	+	+
*	12	CAGGATGGCT TGGAGATGTA C	+	+	+
**	13	AGGATGGCTT GGAGATGTAC T	+	++	++
**	14	GGATGGCTTG AAGATGTACT C	+	++	++
*	15	GATGGCTTGA AGATGTACTC G	+	+	+
*	16	ATGGCTTGAA GATGTACTCG A	+/-	+	+
*	2	TGGCTTGAAG ATGTACTCGA T	++	+	+
**	17	GGCTTGAAGA TGTACTCGAT C	+	++	++
*	18	GCTTGAAGAT GTACTCGATC T	+/-	+	+
	19	CTTGAAGATG TACTCGATCT C	–	+/-	+/-
*	20	GGATGGCTTG AAGATGTACT	+/-	+	+
*	21	GGATGGCTTG AAGATGTAC	+/-	+	+
	22	GGATGGCTTG AAGATGTA	–	–	–
	23	GGCTTGAAGA TGTACTCGAT	–	–	–
	24	GCTTGAAGAT GTACTCGAT	–	–	–
	25	CTTGAAGATG TACTCGAT	–	–	–
	26	TGGCTTGAAG ATGTACTCGA	–	–	–
	27	TGGCTTGAAG ATGTACTCG	–	–	–
	28	GGGCACACAG GATGGCTTGA AGATGTACTCGAT	+/-	+/-	+/-
*	29	GGGCACACAG GATGGCTTGA AGA	+/-	+	+

+ indicates profound inhibition of expression
– indicates no inhibition of expression
+/- indicates some inhibition of expression
The antisense sequences are represented, as per the convention, in the 5'→ 3' orientation. Antisense molecules are complements to the coding strand of the DNA.

EXAMPLE 11

Effect of Antisense Oligonucleotides on Pancreatic Cancer Cells

Vascular endothelial growth factor (VEGF) is overexpressed in human pancreatic cancer (PaCa). Previous studies suggest that VEGF acts not directly on PaCa cells, but as paracrine stimulator of tumor neoangiogenesis. This study investigated VEGF production/ expression in human pancreatic cancer cells and evaluated the effect of a VEGF antisense oligonucleotide on in-vivo growth and angiogenesis of human PaCa in an orthotopic nude mouse model.

In-vitro: Two human PaCa cell lines (AsPC-1 poorly differentiated; HPAF-2, moderately to well differentiated) were evaluated/tested for VEGF mRNA transcripts by RT-PCR. VEGF

secretion in cell culture supernatant was assessed by ELISA. Both PaCa cell lines expressed VEGF mRNA and secreted VEGF protein (AsPC-1: 4205±39 pg/10^6 cells; HPAF-2: 8123±64 pg/10^6 cells).*In-vivo:* VEGF antisense oligonucleotide (AS-3/Veglin-3, SEQ ID NO:2) were synthesized with phosphorothioate modification. 1 mm^3 fragments of sc. PaCa donor tumors were orthotopically implanted into the pancreas of nude mice. Animals received either AS-3 (10 mg/kg, daily) or the vehicle ip. for 14 weeks. Volume of primary tumor (TU-Vol.), metastic spread (Met-Score), and VEGF-expression in serum ($VEGF_S$) and ascites ($VEGF_A$) were determined at autopsy. Microvessel density (MVD) was analyzed by immunohistochemistry in CD31-stained tumor sections. The results of these *in vivo* studies are shown in Table 4.

Table 4. Results of AS-3/Veglin-3 treatment.

	AsPC-1		HPAF-2	
*=p<0.05 vs. Control	Control	AS-3	Control	AS-3
TU-Vol. (mm^3)	1404 ± 149	1046 ± 81	3829 ± 594	860 ± 139*
Met-Score (pts.)	16.7 ± 0.9	6.5 ± 0.8*	8.3 ± 1.5	2.5 ± 0.2*
Survival (n / n)	1 / 8	6 / 8*	4 / 8	7 / 8
$VEGF_S$ (pg/ml)	59.5 ± 5.8	26.6 ± 1.1*	192.3 ± 41.2	38.3 ± 6.1*
$VEGF_A$ (pg/ml)	1190 ± 88	no ascites	1405 ± 97	no ascites
MVD (/0.74 mm^2)	64.1 ± 4.4	33.2 ± 2.3*	76.4 ± 6.0	24.1 ± 2.5*

Human PaCa cells secrete a high level of biologically active VEGF *in vitro.* VEGF-antisense therapy reduces VEGF secretion and tumor neoangiogenesis *in vivo*, thereby reducing tumor growth and metastasis, and improving survival. Metastasis seems to be particularly susceptible to VEGF-AS therapy. None of the AS-3 treated animals developed ascites, suggesting that vascular permeability was also reduced by inhibiting VEGF expression in PaCa cells.

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WHAT IS CLAIMED:

1. A composition for inhibiting vascular endothelial growth factor expression in a cell comprising:

an antisense oligonucleotide directed against vascular endothelial growth factor (VEGF) wherein said antisense oligonucleotide inhibits proliferation of cultured Kaposi's Sarcoma cells at an IC_{50} concentration of less than or equal to about 1.5 micromolar.

2. The composition of claim 1 wherein said antisense oligonucleotide is selected from the group consisting of SEQ ID NOS: 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 21, 28 and 29.

3. The composition of Claim 2 wherein said antisense oligonucleotide is encapsulated in a liposome.

4. The composition of Claim 2 further comprising an antibody that specifically interacts with a vascular endothelial growth factor or a vascular endothelial growth factor receptor.

5. The composition of Claim 4 wherein said antibody is specific for Flt-1.

6. The composition of Claim 4 wherein said antibody is specific for Flk-1.

7. The composition of Claim 2 further comprising a second antisense oligonucleotide chosen from the group consisting of SEQ ID NOS: 1-29.

8. The composition of Claim 7 wherein said first and second oligonucleotides are encapsulated in a liposome.

9. The composition of Claim 1 wherein said antisense oligonucleotide inhibits proliferation of cultured ovarian carcinoma cells at an IC_{50} concentration of less than or equal to about 2 micromolar.

10. The composition of Claim 9 wherein said antisense oligonucleotide is chosen from the group consisting of SEQ ID NOS: 9, 10, 13, 14, 17, 28 and 29.

11. The composition of Claim 1 wherein said antisense oligonucleotide inhibits proliferation of cultured melanoma cells at an IC_{50} concentration of less than or equal to about one micromolar.

12. The composition of Claim 11 wherein said antisense oligonucleotide is chosen from the group consisting of SEQ ID NOS: 12, 13, 14, and 17.

13. The composition of Claim 1 wherein said cells are epithelial cells.

14. The composition of Claim 1 wherein said cells are ovarian cancer cells, melanoma cells, Kaposi's sarcoma cells or pancreatic cancer cells.

15. The composition of Claim 14 wherein said cells are metastatic.

ABSTRACT

The invention provides novel VEGF antagonist/s for use in the treatment of Kaposi's sarcoma (KS), ovarian cancer or melanoma in patients. VEGF antagonists are capable of inhibiting the growth of KS cells in culture by inhibiting the production of VEGF, or by interfering with the binding of VEGF to its cognate receptors or interfere with the biological effects of VEGF. VEGF antagonists of the invention can be antisense oligonucleotides that inhibit VEGF expression. Other VEGF antagonist such as VEGF antibodies, VEGF receptor antibodies, soluble forms of VEGF receptors that bind VEGF away from the cells, or agents that inhibit the signal of VEGF into the cell such as protein kinase inhibitors etc. can also be used. The novel antisense oligonucleotides may also be used to inhibit VEGF and thus new blood vessel formation in diseases such as tumors, proliferative retinopathy, or collagen vascular diseases such as rheumatoid arthritis, and skin diseases such as pemphigus and psoriasis. The KS cell lines also allow the screening of other VEGF inhibitors.

Figure 1

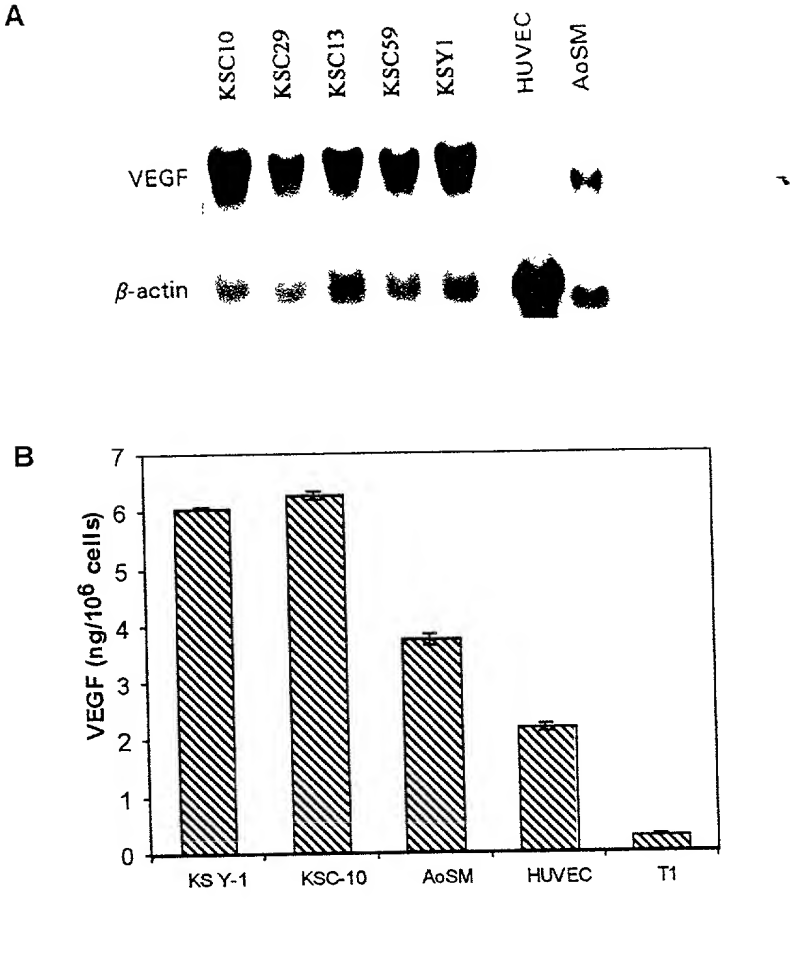
A
KSC10
KSC29
KSC13
KSC59
KSY1
HUVEC
AoSM
VEGF
β-actin
B
VEGF (ng/10^6 cells)
0
1
2
3
4
5
6
7
KS Y-1
KSC-10
AoSM
HUVEC
T1

Figure 2

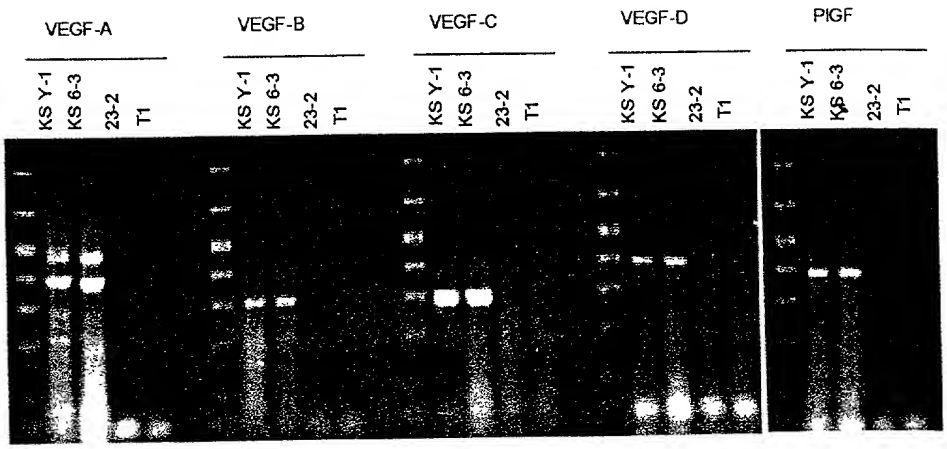

Figure 3

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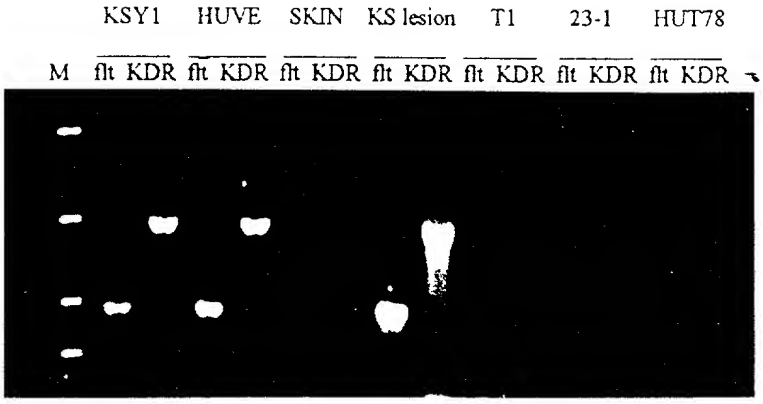
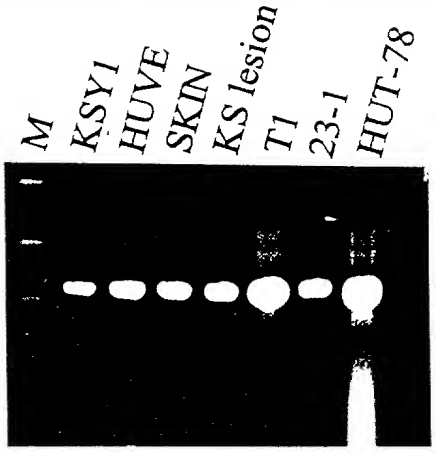

KSY1
HUVE
SKIN
KS lesion
T1
23-1
HUT78
M
flt KDR flt KDR flt KDR flt KDR flt KDR flt KDR flt KDR

B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HUVE
SKIN
KS lesion
T1
23-1
HUT-78

Figure 4

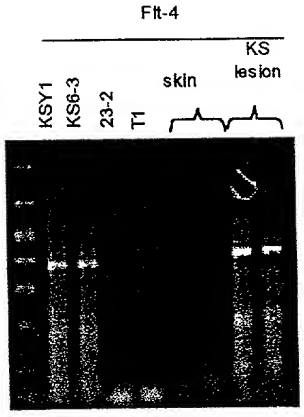
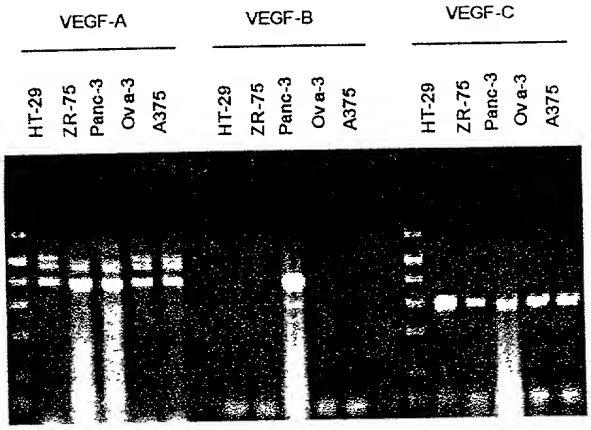


Figure 5

A


VEGF-A
VEGF-B
VEGF-C
HT-29
ZR-75
Panc-3
Ov a-3
A375
HT-29
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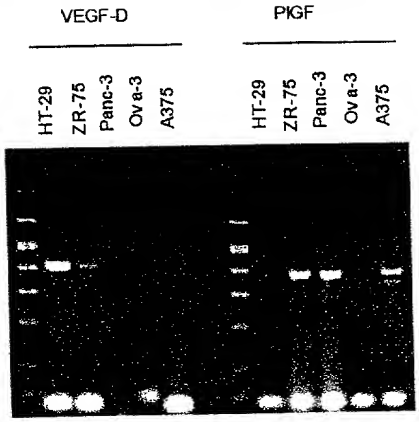
HT-29
ZR-75
Panc-3
Ov a-3
A375

B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PlGF
HT-29
ZR-75
Panc-3
Ov a-3
A375
HT-29
ZR-75
Panc-3
Ov a-3
A375

A

Figure 6A

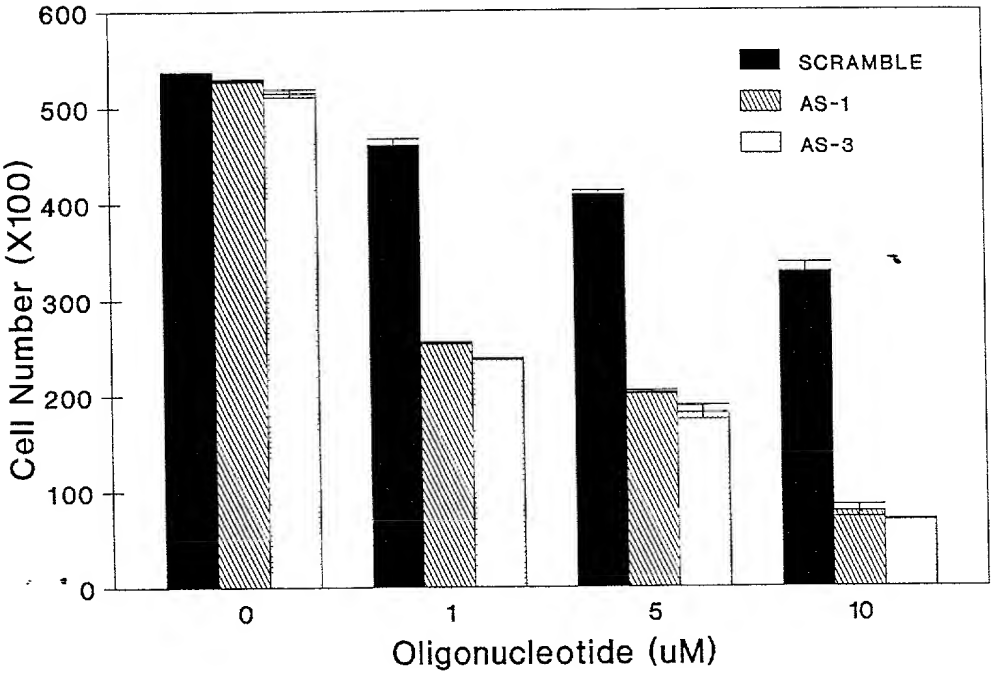
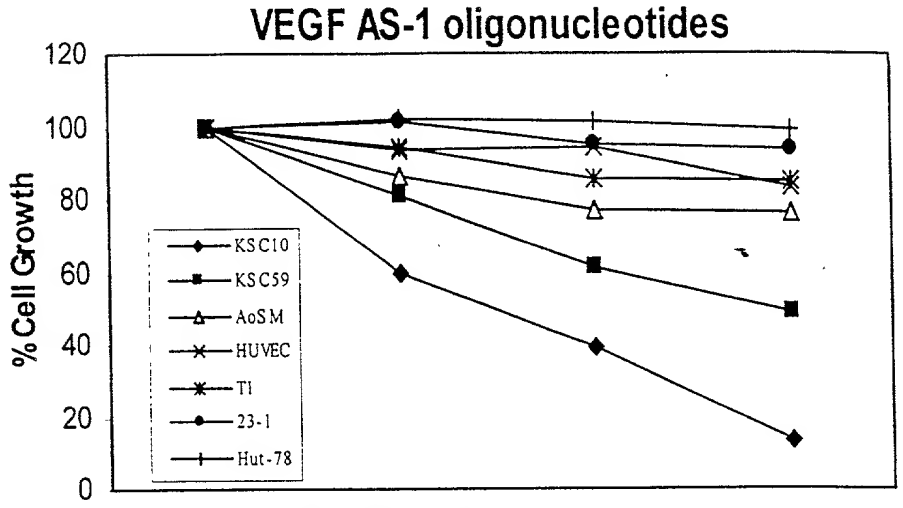

600
500
400
300
200
100
0
Cell Number (X100)
SCRAMBLE
AS-1
AS-3
0
1
5
10
Oligonucleotide (uM)

Figure 6B C D

B



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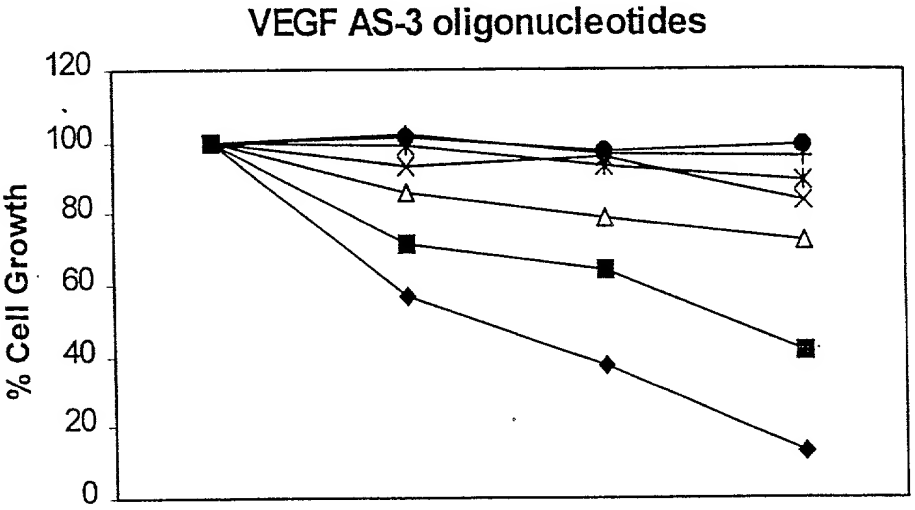

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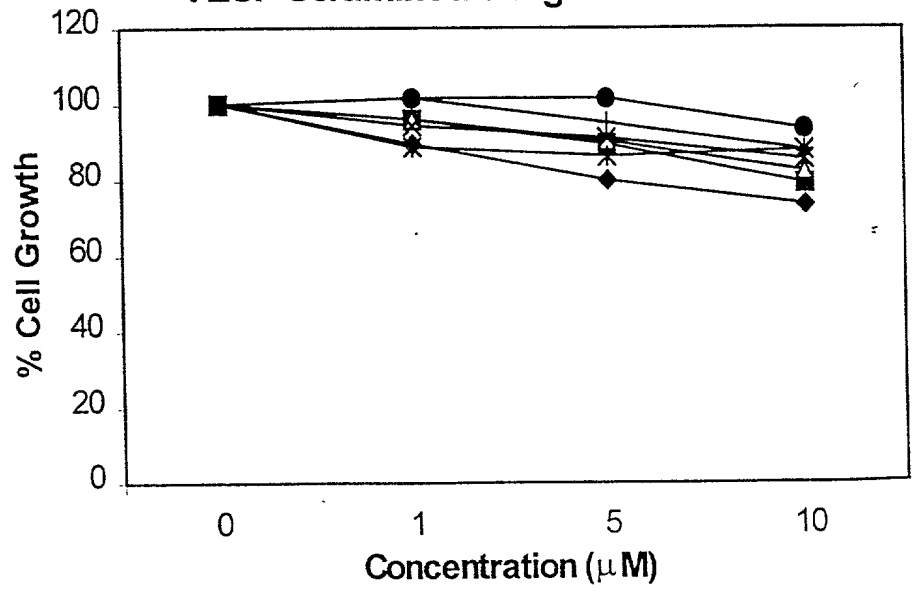
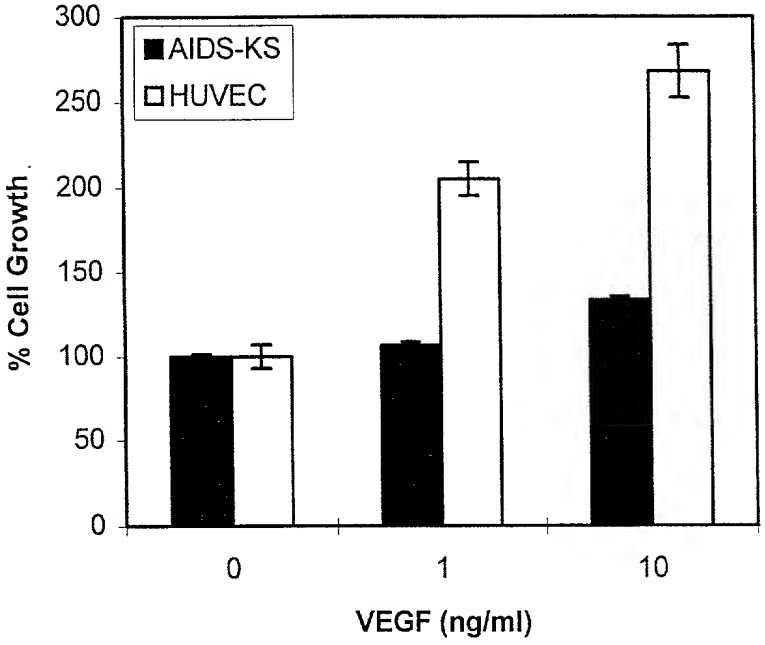


Figure 6E, F

E



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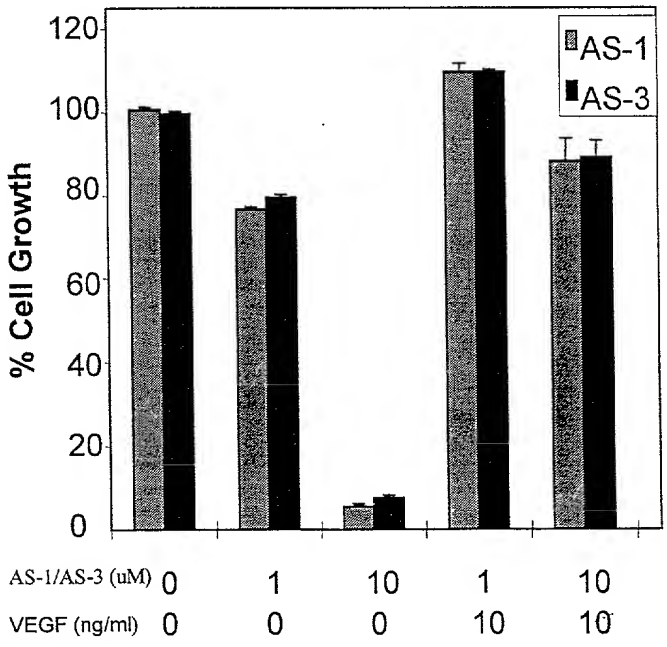
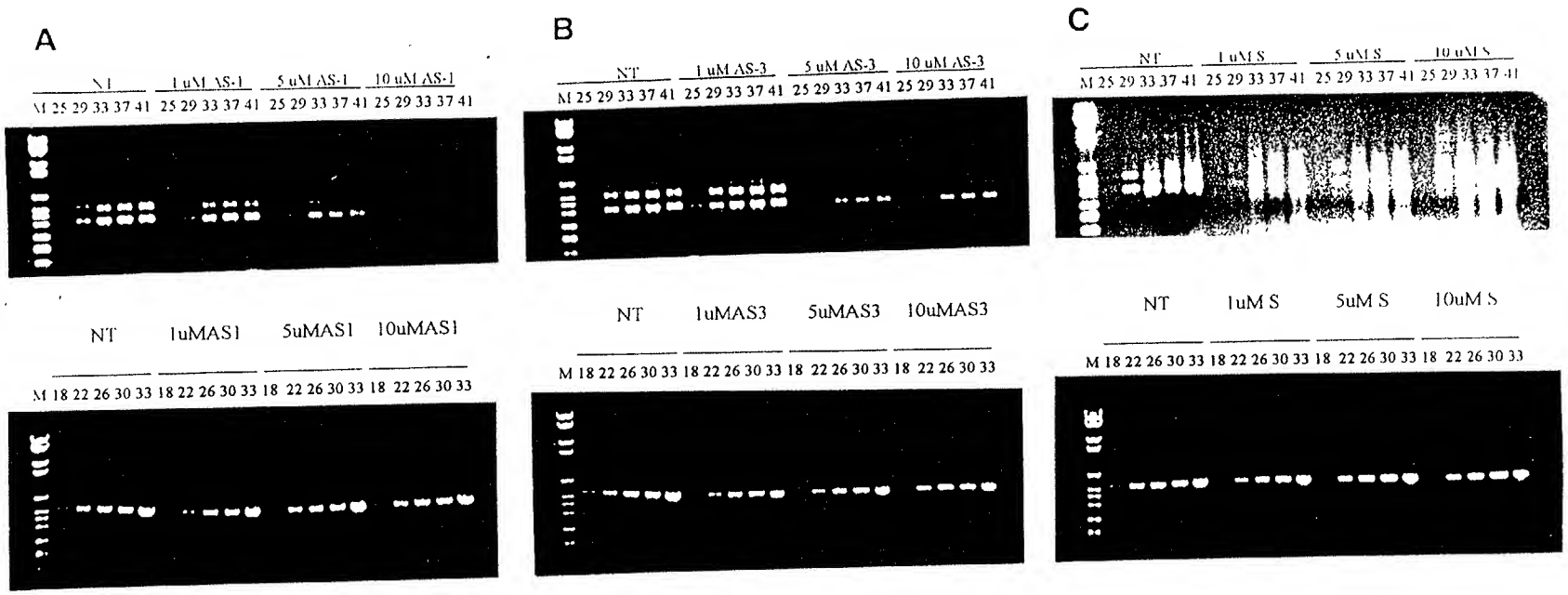


Figure 7A B C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NT
1uM AS-1
5uM AS-1
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NT
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5uMAS1
10uMAS1
M 18 22 26 30 33 18 22 26 30 33 18 22 26 30 33 18 22 26 30 33
B
NT
1uM AS-3
5uM AS-3
10uM AS-3
M 25 29 33 37 41 25 29 33 37 41 25 29 33 37 41 25 29 33 37 41
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5uMAS3
10uMAS3
M 18 22 26 30 33 18 22 26 30 33 18 22 26 30 33 18 22 26 30 33
C
NT
1uM S
5uM S
10uM S
M 25 29 33 37 41 25 29 33 37 41 25 29 33 37 41 25 29 33 37 41
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5uM S
10uM S
M 18 22 26 30 33 18 22 26 30 33 18 22 26 30 33 18 22 26 30 33

D

Figure 7D

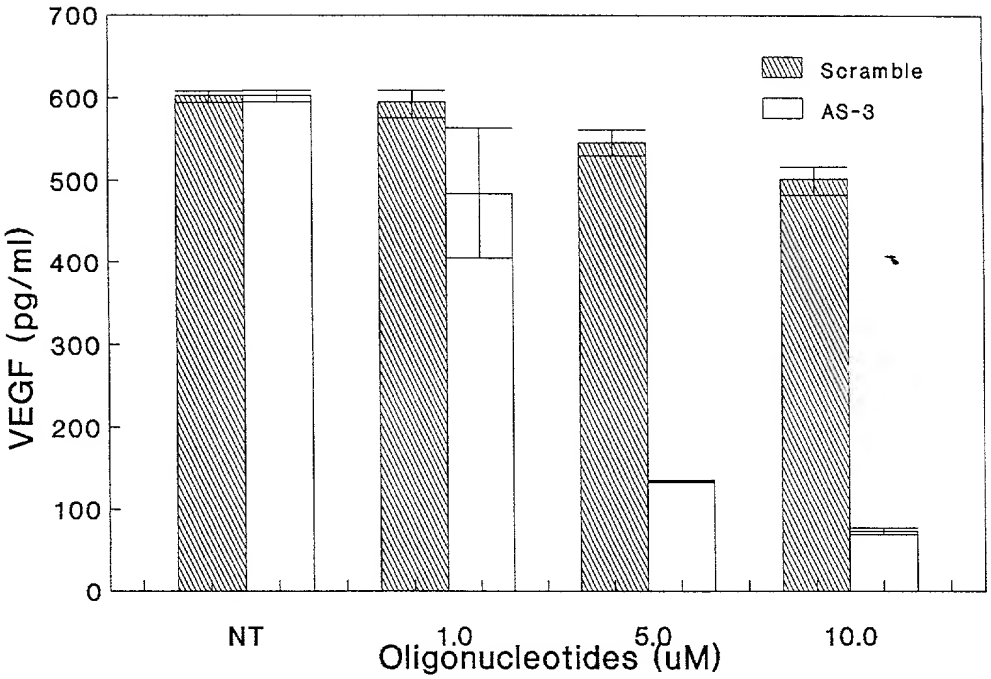
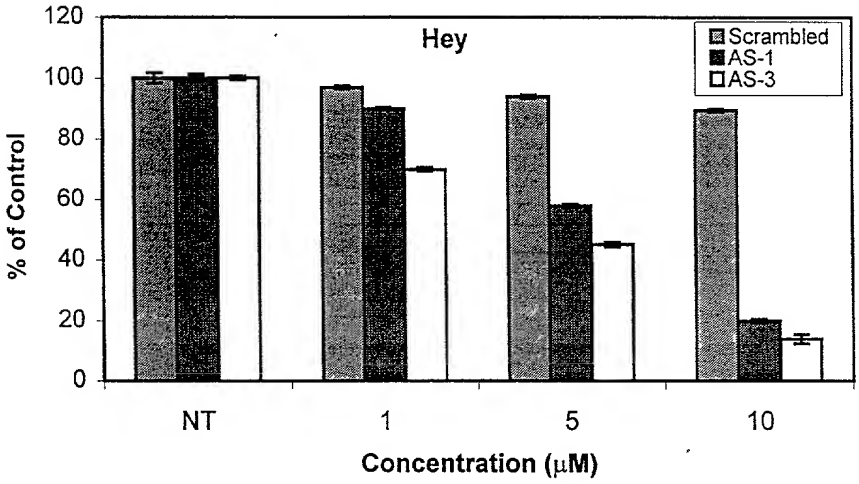


Figure 7D

Figure 7E


Hey
Scrambled
AS-1
AS-3
% of Control
120
100
80
60
40
20
0
NT
1
5
10
Concentration (μM)

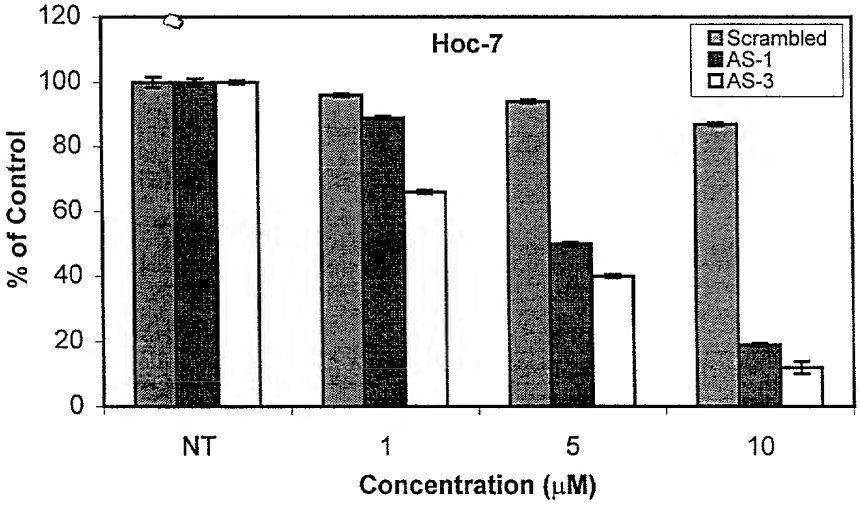
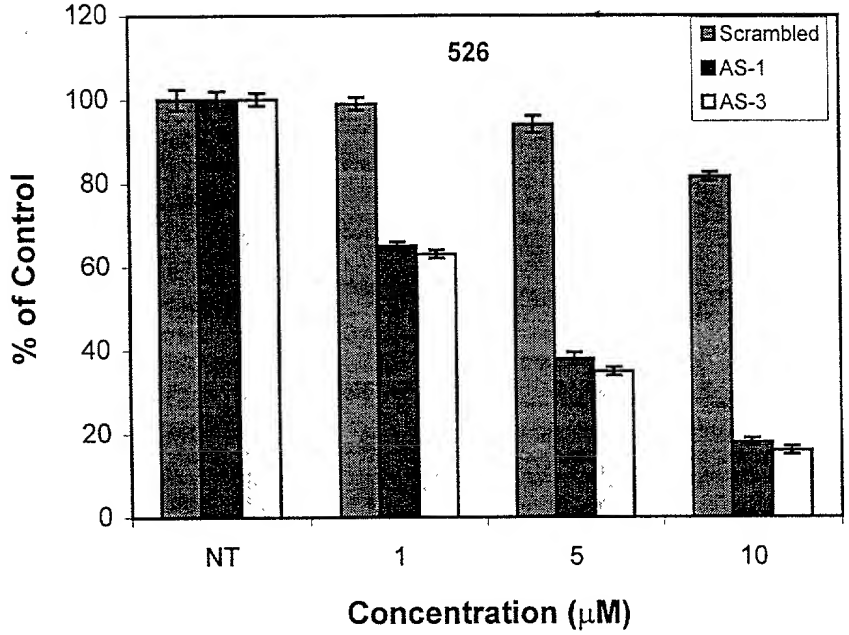

Hoc-7
Scrambled
AS-1
AS-3
% of Control
120
100
80
60
40
20
0
NT
1
5
10
Concentration (μM)

Figure 7F


526
Scrambled
AS-1
AS-3
% of Control
120
100
80
60
40
20
0
NT
1
5
10
Concentration (μM)

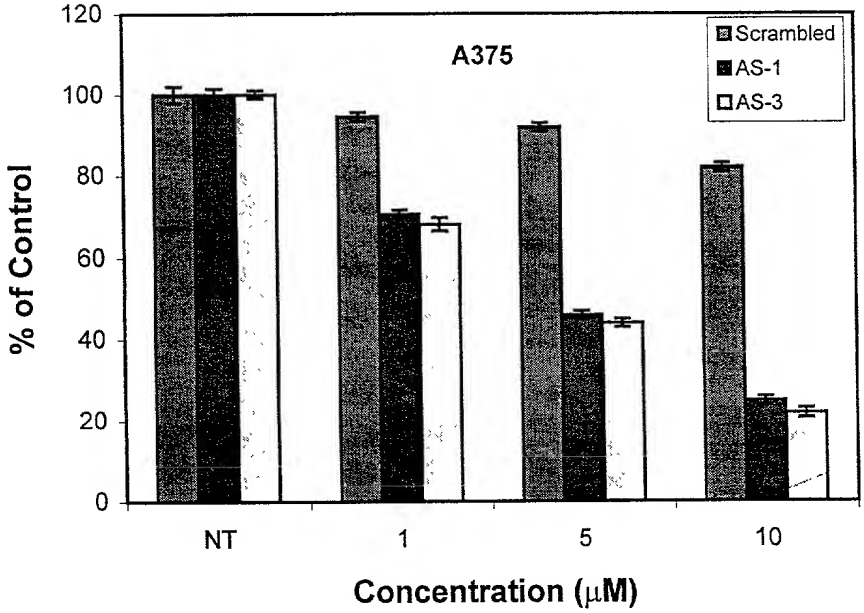

A375
Scrambled
AS-1
AS-3
% of Control
120
100
80
60
40
20
0
NT
1
5
10
Concentration (μM)

Figure 8

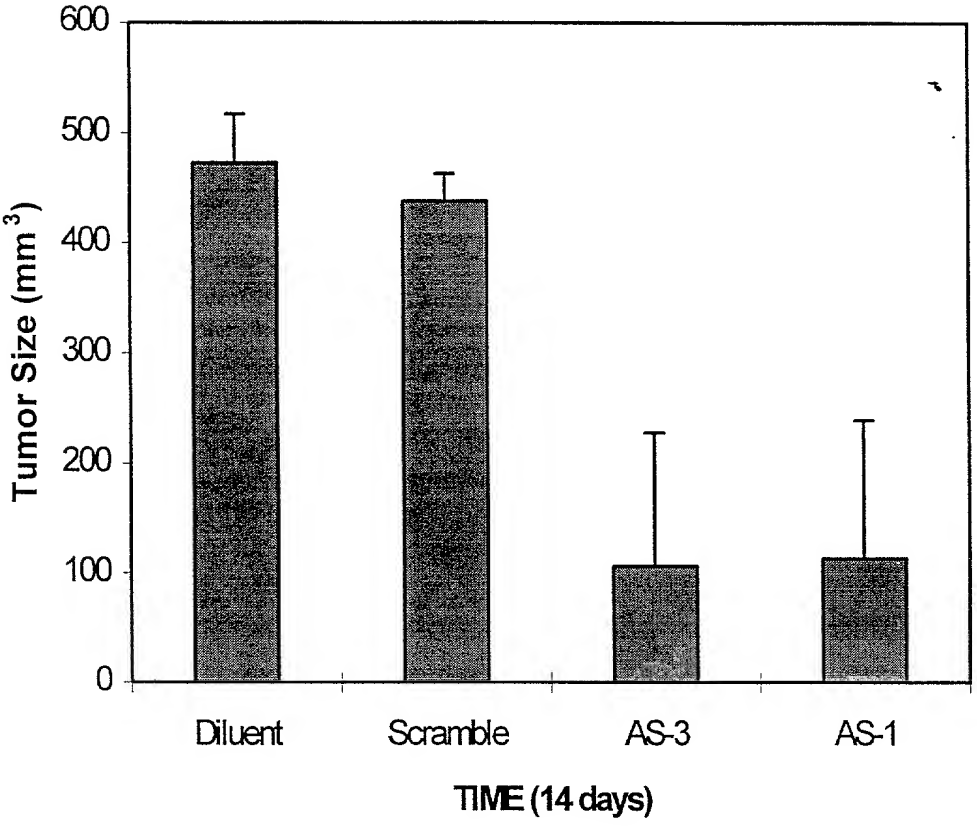
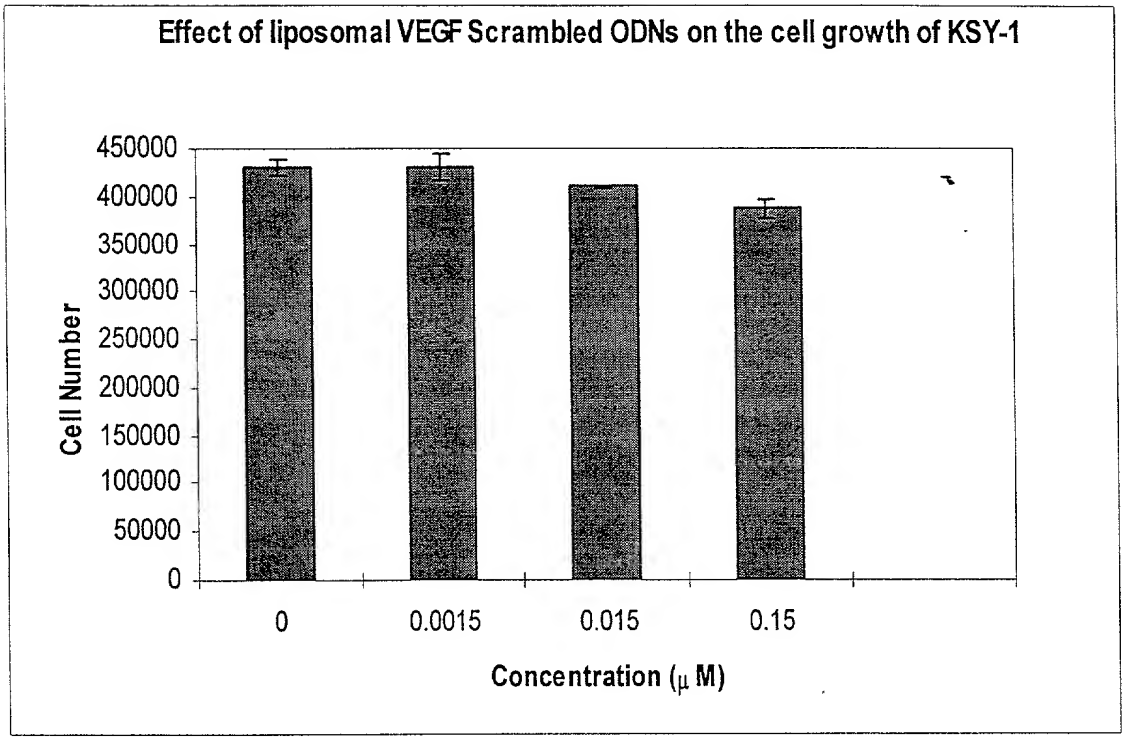

600
500
400
300
200
100
0
Tumor Size (mm3)
Diluent
Scramble
AS-3
AS-1
TIME (14 days)

Figure 9


Effect of liposomal VEGF Scrambled ODNs on the cell growth of KSY-1
Cell Number
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
0
0.0015
0.015
0.15
Concentration (μ M)

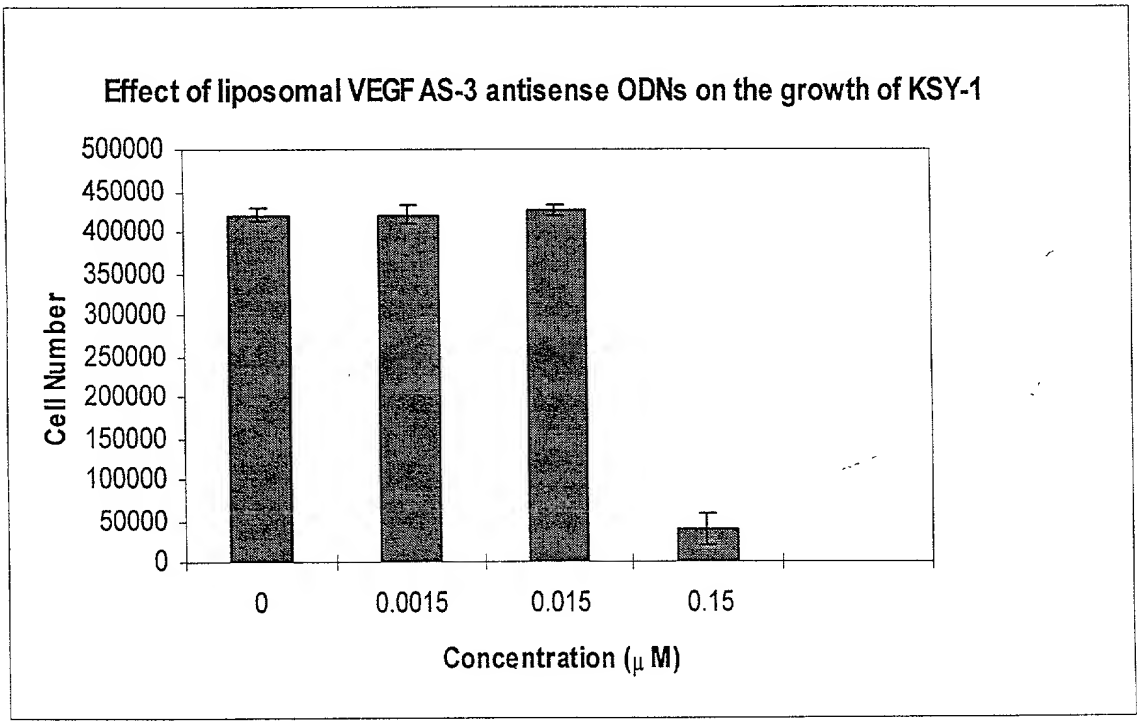
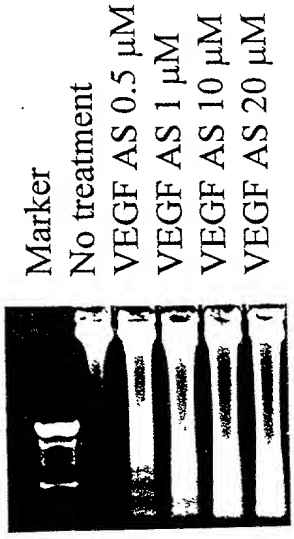

Effect of liposomal VEGF AS-3 antisense ODNs on the growth of KSY-1
Cell Number
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
0
0.0015
0.015
0.15
Concentration (μ M)

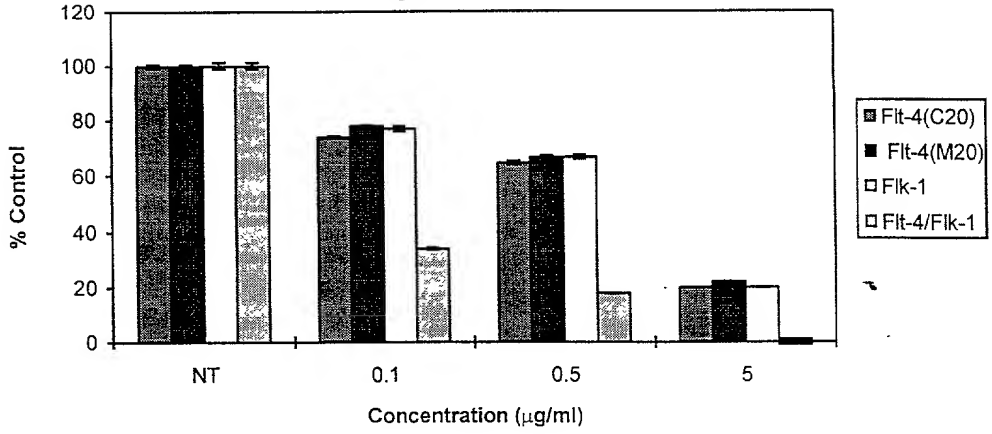
Figure 10


Marker
No treatment
VEGF AS 0.5 µM
VEGF AS 1 µM
VEGF AS 10 µM
VEGF AS 20 µM


Marker
No treatment
VEGF S 0.5 µM
VEGF S 1 µM
VEGF S 10 µM
VEGF S 20 µM

Figure 11

A


120
100
80
60
40
20
0
% Control
NT
0.1
0.5
5
Concentration (μg/ml)
Flt-4(C20)
Flt-4(M20)
Flk-1
Flt-4/Flk-1

B

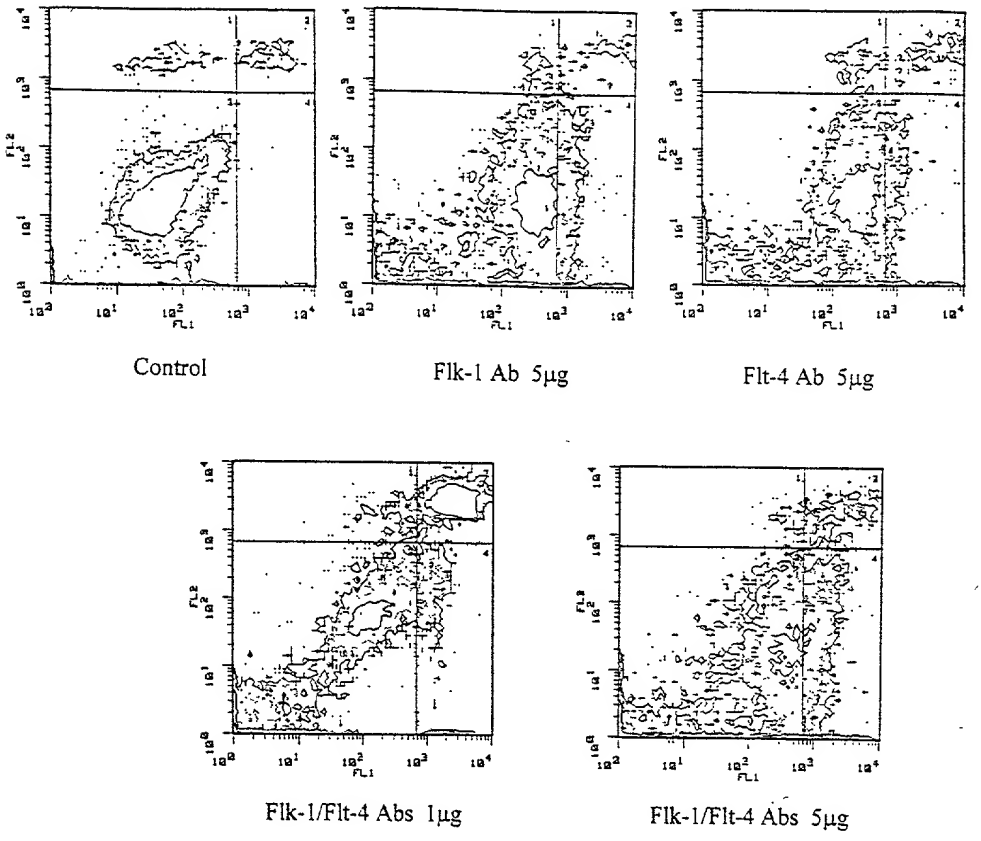

Control
Flk-1 Ab 5μg
Flt-4 Ab 5μg
Flk-1/Flt-4 Abs 1μg
Flk-1/Flt-4 Abs 5μg

Figure 12

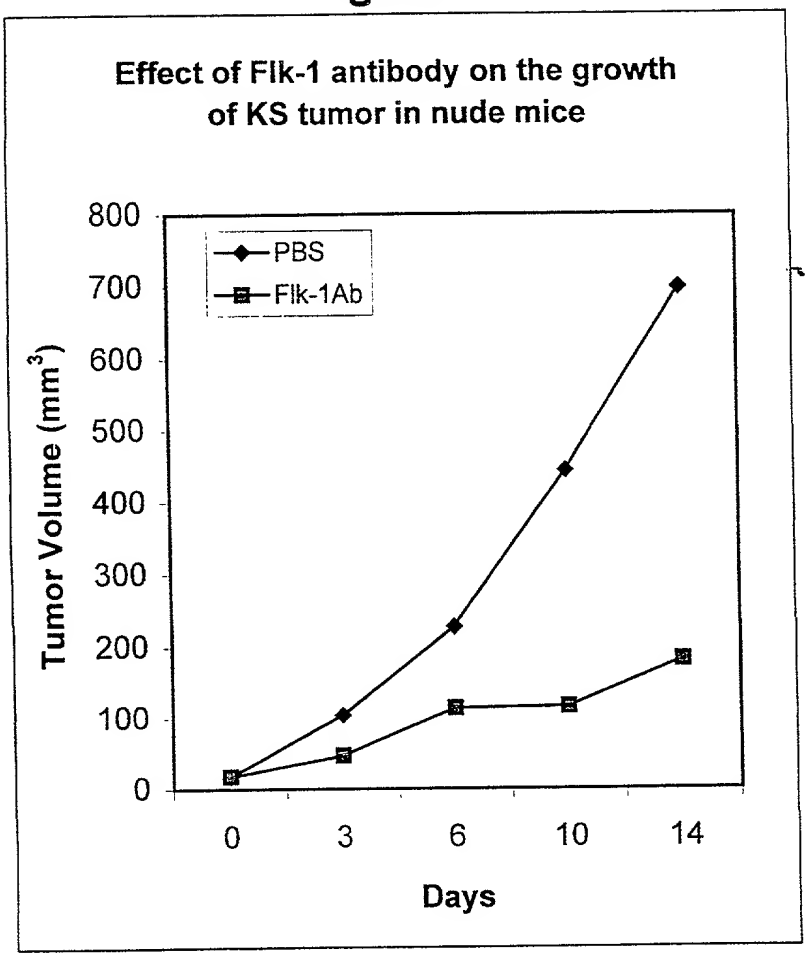

Effect of Flk-1 antibody on the growth of KS tumor in nude mice
Tumor Volume (mm3)
800
700
600
500
400
300
200
100
0
PBS
Flk-1Ab
0
3
6
10
14
Days

Figure 13

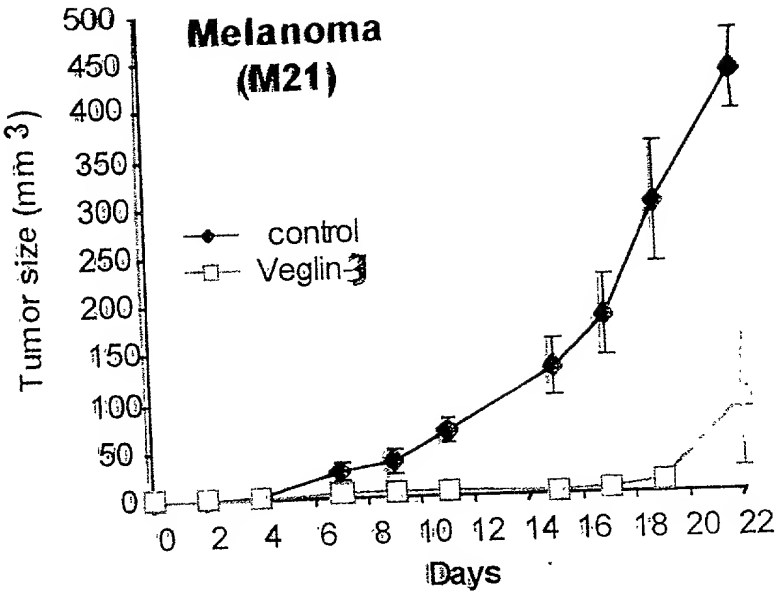
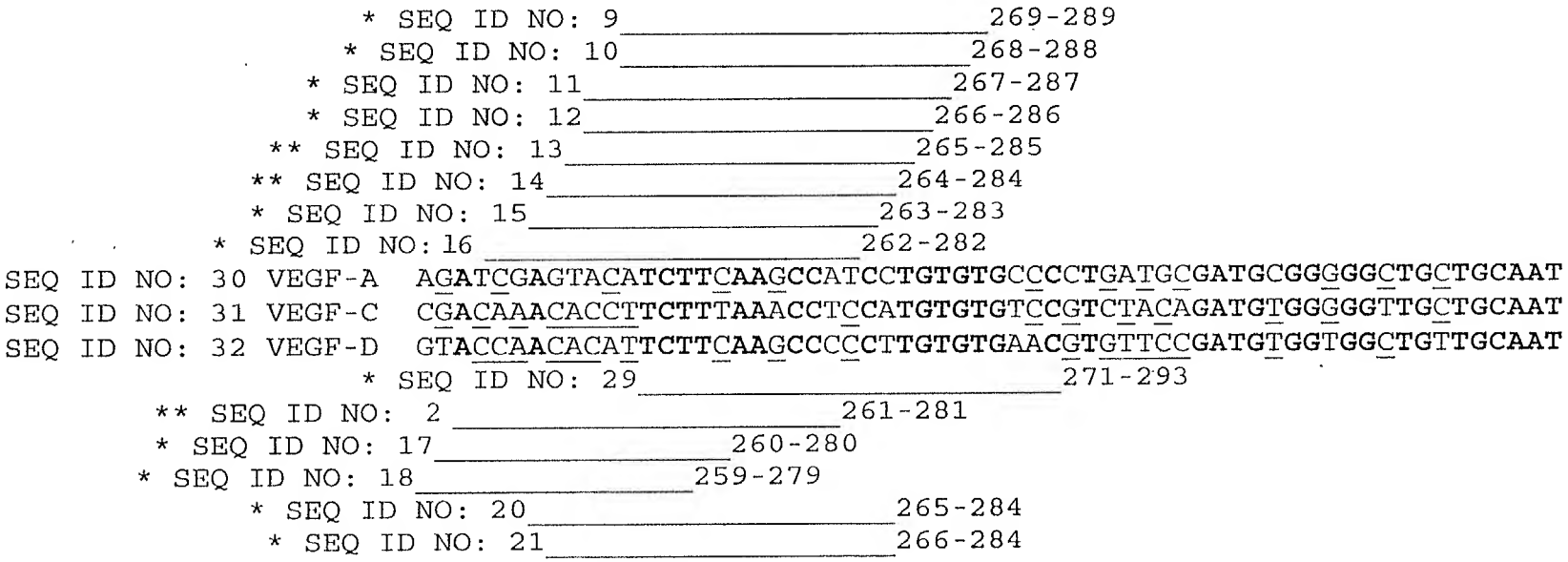

Melanoma
(M21)
Tumor size (mm 3)
500
450
400
350
300
250
200
150
100
50
0
control
Veglin-3
0 2 4 6 8 10 12 14 16 18 20 22
Days

FIGURE 14


* SEQ ID NO: 9 269-289
* SEQ ID NO: 10 268-288
* SEQ ID NO: 11 267-287
* SEQ ID NO: 12 266-286
** SEQ ID NO: 13 265-285
** SEQ ID NO: 14 264-284
* SEQ ID NO: 15 263-283
* SEQ ID NO: 16 262-282
SEQ ID NO: 30 VEGF-A AGATCGAGTACATCTTCAAGCCATCCTGTGTGCCCCTGATGCGATGCGGGGGCTGCTGCAAT
SEQ ID NO: 31 VEGF-C CGACAAACACCTTCTTTAAACCTCCATGTGTGTCCGTCTACAGATGTGGGGGTTGCTGCAAT
SEQ ID NO: 32 VEGF-D GTACCAACACATTCTTCAAGCCCCCTTGTGTGAACGTGTTCCGATGTGGTGGCTGTTGCAAT
* SEQ ID NO: 29 271-293
** SEQ ID NO: 2 261-281
* SEQ ID NO: 17 260-280
* SEQ ID NO: 18 259-279
* SEQ ID NO: 20 265-284
* SEQ ID NO: 21 266-284

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As below named inventors,

We hereby declare that our residence, post office address and citizenship are as stated below next to our names.

We believe we are the original, joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled: METHOD AND COMPOSITION FOR TREATMENT OF KAPOSI'S SARCOMA, the specification of which

__X__ is attached hereto.

_____ was filed on [date] and assigned Application Serial Number [ ] and was amended on [date].

We hereby state that we have reviewed and understand the contents of the above-identified specification, including the claims as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a).

We hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign applications for patent or inventor's certificate, listed below and so identified, and we have also identified below any foreign application for patent or inventor's certificate on this invention filed by us or our legal representatives or assigns and having a filing date before that of the application on which priority is claimed.

Number	Country	Day/Month/ Year Filed	Priority Claimed - Yes or No

We hereby claim the benefit under Title 35, United States Code, § 120 of any United States applications listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, we acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

Application Serial No.	Filing Date	Status
09/016,541	January 30, 1998	Pending

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States provisional application in the manner provided by the first paragraph of Title 35, United States Code, § 112, we acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior provisional application and the filing date of this application.

Provisional Application No.	Filing Date	Status
60/037,004	January 31, 1997	Abandoned

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

We hereby appoint the following attorneys and agents, with full power of substitution and revocation, to prosecute this application and to transact all business in the United States Patent and Trademark Office connected therewith and request that all correspondence and telephone calls with respect to this application be directed to McCUTCHEN, DOYLE, BROWN & ENERSEN, LLP Three Embarcadero Center, San Francisco, California 94111, Telephone No. (415) 393-2000:

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